(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 July 2001 (19.07.2001)

PCT

(10) International Publication Number
WO 01/51610 A1

(51) International Patent Classification[7]: C12N 5/06, 5/08, 15/12

(21) International Application Number: PCT/AU01/00029

(22) International Filing Date: 12 January 2001 (12.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PQ 5098	14 January 2000 (14.01.2000)	AU
PQ 7045	20 April 2000 (20.04.2000)	AU
PQ 7143	27 April 2000 (27.04.2000)	AU

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/51610 A1

(54) Title: ECTODERM CELL PRODUCTION

(57) Abstract: A method of producing definitive ectoderm equivalent (DEE) cells, which method includes providing a source of early primitive ectoderm-like (EPL) cells; a conditioned medium as hereinbefore defined; or an extract therefrom exhibiting neural inducing properties; and contacting the EPL cells with the conditioned medium, for a time sufficient to generate controlled differentiation to neurectoderm cells.

ECTODERM CELL PRODUCTION

The present invention relates to definitive ectoderm equivalent cells produced *in vitro,* to marker genes, including novel marker genes, for definitive ectoderm and to differentiated cells or partially differentiated cells derived therefrom. The present invention also relates to methods of producing, differentiating and culturing the cells of the present invention, and to uses thereof.

Initial developmental events within the mammalian embryo entail the elaboration of extra-embryonic cell lineages and result in the formation of the blastocyst, which comprises trophectoderm, primitive endoderm and a pool of pluripotent cells, the inner cell mass (ICM/epiblast). As development continues, the cells of the ICM/epiblast undergo rapid proliferation, selective apoptosis, differentiation and reorganisation as they develop to form the primitive ectoderm. In the mouse, the cells of the ICM begin to proliferate rapidly around the time of blastocyst implantation. The resulting pluripotent cell mass expands into the blastocoelic cavity. Between 5.0 and 5.5 days post coitum (dpc) the inner cells of the epiblast undergo apoptosis to form the proamniotic cavity. The outer, surviving cells, or early primitive ectoderm, continue to proliferate and by 6.0-6.5 dpc have formed a pseudo-stratified epithelial layer of pluripotent cells, termed the primitive or embryonic ectoderm. Primitive ectoderm cells are pluripotent, and distinct from cells of the ICM, giving rise to the germ cells and acting as a substrate for the generation of the primary germ layers of the embryo proper (mesoderm, endoderm and ectoderm) and the extra-embryonic mesoderm during gastrulation.

By 4.5 dpc pluripotent cells exposed to the blastocoelic cavity have differentiated to form primitive endoderm. The primitive endoderm gives rise to two distinct endodermal cell populations, visceral endoderm, which remains in contact with the epiblast, and parietal endoderm, which migrates away from the pluripotent cells to form a layer of endoderm adjacent to the trophectoderm. Formation of these endodermal layers is coincident with the formation of primitive ectoderm and the creation of an inner cavity.

In the human and in other mammals, formation of the blastocyst, including development of ICM cells and their progression to pluripotent cells of the primitive ectoderm and subsequent differentiation to form the embryonic germ layers, follow a similar developmental process.

The transition of ICM cells to primitive ectoderm represents a differentiation event whereby the cell population which comprises the primitive ectoderm is distinct from that of the ICM. Cells of the primitive ectoderm are no longer totipotent, being limited in their differentiation potential in that they are unable to form some extra-embryonic lineages, such as trophectoderm (Gardner and Rossant 1979). Furthermore, the primitive ectoderm exhibits a higher rate of cell division than the ICM (Snow 1977) and the repertoire of genes expressed is distinct. *Rex1* and *Gbx2* are expressed exclusively in the ICM (Chapman et al. 1997; Rogers et al. 1991) whereas *Fgf5* is up regulated upon conversion to primitive ectoderm (Haub and Goldfarb 1991; Hébert et al. 1991).

During gastrulation the primitive ectoderm is converted into a trilaminar structure, consisting of ectoderm, mesoderm and an inner layer of endoderm. These primary germ layers form a variety of tissues which embody the basic design of the foetus. The onset of gastrulation is marked by appearance of the primitive streak at 6.5 dpc in the primitive ectoderm. The mesodermal cells emerging from the primitive streak migrate laterally towards the anterior end of the embryo between the epiblast basal surface and the visceral endoderm. It is the differentiation of this mesoderm germ layer which results in the formation of vertebrae, ribs, dermis, kidney and muscle tissues in the developing foetus and adult.

Cells that remain in the anterior region and distal of the primitive ectoderm give rise to definitive ectoderm (Beddington 1982), from which both the neurectoderm and surface ectoderm are derived (Lawson and Pederson 1987; Tam 1989).

Neurectoderm is the progenitor tissue of the central and peripheral nervous system and is generated from cells in the dorsal region of the definitive ectoderm

(Weinstein et al. 1997). Neuralization is initiated at 7.5 dpc when the ectoderm in this region becomes flattened and develops a thick ridge around its periphery to form the neural plate. The neural tube is produced by an infolding of the neural plate, whose lateral margins meet and then fuse at the dorsal mid-line. At the time of closure, the neural tube consists of a single layer of neuroepithelial cells which forms neural precursor cells and the neural crest. Together, these cells constitute the predecessors of the nervous system (Beddington 1983).

Cells in the ventral side of the definitive ectoderm follow a different developmental pathway that leads to the formation of surface ectoderm (Hemmati-Brivanlou and Melton 1997). The epidermis, lens and cornea of the vertebrate foetus are derived from the surface ectoderm which consists of a single epithelial layer (Ede 1978).

The factors which induce patterning of the definitive ectoderm to form either surface ectoderm or neurectoderm, are largely unknown. Over the past five years however, a large body of evidence has come to support a 'default' model of neural induction (see Weinstein and Hemmati-Brivanlou 1999). The model argues that the differentiation of surface ectoderm requires inductive signals, while neuralization of the dorsal ectoderm requires only an inhibition of this signalling.

A number of genes have been identified which are transiently and spatially expressed within the developing cell populations of the mouse embryo. The expression profiles of *Oct4* (Rosner et al. 1990; Schöler et al. 1990a), *Rex1* (Rogers et al. 1991), *Gbx2* (Bulfone et al. 1993; Chapman et al. 1997) and *Fgf5* (Haub and Goldfarb 1991; Hébert et al. 1991) and *Sox1* (Pevney et al, 1998) have all been documented in developing pluripotent cell populations of the embryo.

Precise analysis of gene expression during early mouse embryogenesis has, however, been hindered by the small size, complexity and inaccessibility of the mouse embryo within the uterine environment. As a consequence, the onset and cessation of gene expression has rarely been mapped closely and early intermediates of the ectodermal lineage have not been defined by gene expression. In particular, while markers for the ICM (*Rex1*), primitive ectoderm

(FGF5) and early neurectoderm (*Sox1*)have been described, markers for definitive ectoderm have not.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In a first aspect of the present invention it has been found that by growing early primitive ectoderm-like (EPL) cells formed in suspension culture in the presence of factors in a conditioned medium MEDII, for a time insufficient to form neurectoderm cells, cells exhibiting characteristics of definitive ectoderm may be produced. Definitive ectoderm cells express lower levels of *Oct4* than pluripotent cells, and do not express neural lineage markers.

Accordingly, there is provided a method for preparing definitive ectoderm equivalent (DEE) cells *in vitro*, which method includes

providing

a source of early primitive ectoderm-like (EPL) cells; and

a conditioned medium as hereinafter described; or an extract therefrom exhibiting neural inducing properties; and

culturing the EPL cells in the conditioned medium or extract for a time sufficient to permit controlled differentiation to definitive ectoderm equivalent cells.

The protocol used to induce neurectoderm-specific differentiation from ES cells may be similar to that described in Australian patent application PQ7143, to applicants, the entire disclosure of which is incorporated herein by reference. ES cells may be cultured as aggregates on non-gelatinised bacterial dishes in incomplete ES cell media supplemented with conditioned media, e.g. 50% MEDII for seven days. The Embryoid Bodies cultured in MEDII (EBMs) may be passaged, e.g. every 3-4 days and the conditioned medium replenished e.g. every second day. Day 0 (EBM^0) represents undifferentiated ES cells. Gene expression is assayed daily. In later EBMs the pseudostratified columnar epithelial morphology of neurectoderm is visible as disclosed in an international patent application, to BresaGen Limited, filed conterminously herewith, the full disclosure of which is incorporated herein by reference (the co-filed international

application). These later EBMs also express the neurectoderm marker *Sox1*. This technique may be used to generate a temporal description of differentiation events with respect to morphology and gene expression, allowing the correlation of distinct cell populations *in vitro* with specific timing of embryonic events *in vivo* and determination of the window of existence for definitive ectoderm.

Applicants have surprisingly found that the definitive ectoderm equivalent (DEE) cells form a distinct cell population.

The conditioned medium utilised in the method according to the present invention is described in International patent application PCT/AU99/00265, to Applicants, the entire disclosure of which is incorporated herein by reference.

The term "conditioned medium" includes within its scope a fraction thereof including medium components below approximately 5 kDa, and/or a fraction thereof including medium components above approximately 10 kDa

Preferably the conditioned medium is prepared using a hepatic or hepatoma cell or cell line, more preferably a human hepatocellular carcinoma cell line such as Hep G2 cells (ATCC HB-8065) or Hepa-1c1c-7 cells (ATCC CRL-2026), primary embryonic mouse liver cells, primary adult mouse liver cells, or primary chicken liver cells, or an extraembryonic endodermal cell or cell line such as the cell lines END-2 and PYS-2. However, the biologically active factor may be isolated from a medium conditioned by liver or other cells from any appropriate species, preferably mammalian or avian. Alternatively, the activity may also be derived by contributions from two or more different conditioned media from cells which express one or the other of the components. The conditioned medium MEDII is particularly preferred.

As stated above, a neural inducing extract from the conditioned medium may be used in place of the conditioned medium. The term "neural inducing extract" as used herein includes within its scope a natural or synthetic molecule or molecules which exhibit(s) similar biological activity, e.g. a molecule or molecules

which compete with molecules within the conditioned medium that bind to a receptor on EPL cells responsible for neural induction.

In one form, the method may include the preliminary steps of including providing

a source of pluripotent cells,

a source of a biologically active factor including

a low molecular weight component selected from the group consisting of proline and peptides including proline and functionally active fragments and analogues thereof; and

a large molecular weight component selected from the group consisting of extracellular matrix proteins and functionally active fragments or analogues thereof, or the low or large molecular weight component thereof; and

contacting the pluripotent cells with the biologically active factor, or the large or low molecular weight component thereof, or the conditioned medium, or the extracellular matrix and/or the low molecular weight component, to produce early primitive ectoderm-like (EPL) cells.

The source of the biologically active factor includes a partially or substantially purified form of the biologically active factor, a conditioned medium including the low and/or large molecular weight component thereof; or an extracellular matrix including at least a large molecular weight component thereof, as described in WO99/53021.

The pluripotent cells from which the EPL cells may be derived, may be selected from one or more of the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation or by nuclear transfer. EPL cells may also be derived from differentiated cells by dedifferentiation.

The step of contacting the pluripotent cells with the biologically active factor, etc. to produce EPL cells may be conducted in any suitable manner. For

example, EPL cells may be generated in adherent culture or as cell aggregates in suspension culture. It is particularly preferred that the EPL cells are produced in suspension culture in a culture medium such as Dulbecco's Modified Eagles Medium (DMEM), supplemented with the biologically active factor etc. It is also preferred that there is little or no disruption of cell to cell contact (i.e. trypsinisation).

In a further preferred form, the method includes the further step of

identifying the DEE cells by procedures including gene expression profiles and differentiation potential.

The conversion of EPL cells to DEE cells may be characterised by

down regulation of *Oct4* expression relative to embryonic stem (ES) cells;

substantial absence of neurectoderm marker expression; and

up regulation of expression of DEE marker genes as hereinafter described.

The conversion of EPL cells to DEE cells may be further characterised by a substantial absence of *Rex*, an ES cell marker.

Specific gene expression profiles identified a period between EBM^5 and EBM^7, corresponding to the loss of high *Oct4* gene expression at day 5 and up regulation of expression of the neurectoderm marker *Sox1*, at day 7, in which a definitive ectoderm-like cell population may be present.

The formation of this distinct cell population is accompanied by the onset of specific gene expression. The identification of differentially expressed genes within this period therefore generates molecular markers for definitive ectoderm.

Accordingly, in a further aspect of the present invention, there is provided a definitive ectoderm equivalent (DEE) cell derived *in vitro*.

It is postulated that the novel intermediate cell population formed during the differentiation of early primitive ectoderm-like (EPL) cells to neurectoderm *in vitro*, is representative of embryonic definitive ectoderm. The DEE cell may be further

characterised in being capable of differentiation into neurectoderm or surface ectoderm cells, but not mesoderm cells.

An *in vitro* differentiation system involving the differentiation of ES cells to a homogenous population of neurectoderm via a primitive ectoderm intermediate is described in the co-filed International application, above. The *in vitro* differentiation system represents a model of the ICM to neurectoderm transition *in vivo*. Specific gene expression profiles of the entire transition period examined by Northern blot, RNase protection and in situ hybridisation analysis identified a period of 2/3 days, between the loss of high *Oct4* gene expression at day 5 and the up regulation of the earliest known neurectodermal marker *Sox1*, at day 7, in which transition to a novel intermediate cell population was postulated.

It is further postulated that the formation of a distinct cell type was likely to be accompanied by the onset of specific gene expression. Accordingly, detailed changes in gene expression using the technique of differential display PCR were examined. This approach revealed the up regulation of at least nineteen genes between days 4 and 6, and the down regulation of a number of genes upon the establishment of neurectoderm as defined by *Sox1* up regulation. This suggested the formation of a distinct cell population at this time. Investigation of the developmental potential of this uncharacterised cell population demonstrated the potential to form either surface ectoderm or neurectoderm, but not mesoderm. This characteristic is attributed to definitive ectoderm in the embryo and indicated formation of a definitive ectoderm equivalent *in vitro*, confirming the existence of this cell type in mammals.

The definitive ectoderm equivalent (DEE) cell according to this aspect of the present invention may accordingly exhibit

down regulation of *Oct4* expression relative to ES cells;
substantial absence of neurectoderm marker expression; and
expression of DEE marker genes.

Applicant has for the first time identified marker genes, including novel marker genes, for definitive ectoderm.

Accordingly, in a further aspect of the present invention, there is provided a marker gene for embryonic definitive ectoderm or a definitive ectoderm equivalent (DEE) cell. Each marker gene may exhibit expression in DEE cells. Each marker gene may exhibit up regulation of expression on day 4, 5 or 6 after initiation of culturing of ES cells in conditioned medium, as hereinbefore described, and/or down regulation of expression on day 7 or 8 after initiation of culturing.

Preferably the DEE marker gene is selected from one or more of the group consisting of known genes including CD24Q, DBI, $P12^{DOC-1}$, Rp123, and novel genes A1712, A17113A, A091, A191, A193 and A205 and gene products thereof, and functionally active fragments or analogues thereof, and molecules which compete therewith for biological activity.

Of the gene markers described above, six sequences are novel sequences. Accordingly in a still further aspect of the present invention there is provided a gene sequence selected from one or more of the group consisting of A1712, A17113A, A091, A191, A193 and A205 and functionally active fragments or analogues thereof and molecules which compete therewith for biological activity.

The gene markers described herein may be isolated by screening for specific transcripts with expression within the definitive ectoderm cell population postulated to be present during days 5-7, preferably excluding transcripts expressed in ES, EPL and neurectoderm cells. In order to achieve this, the gene expression of EBM^0 to EBM^9 may be compared by ddPCR. The EBM^{0-9} series was generated and is described in detail below.

RNA samples may be reverse transcribed with an anchored oligo-dT_{12} primer and first strand cDNA was PCR amplified with the oligo-dT_{12} primer in combination with one of the six different arbitrary 5' primers (OPERON primer kits *OPA and OPB*). *Figures 4, 5 and 6 below show the ddPCR profile of* EBM^{0-9} using the OPB-07, OPB-09 and OPA-17 5' oligonucleotides respectively. Each duplicated banding profile was generated from independent reverse transcriptions, demonstrating consistency in amplification and detection of differential gene expression during differentiation. Furthermore, samples were re-run on a second

denaturing gel and displayed the same expression profiles (data not shown). Amplified products were labelled according to the arbitrary 5' primer used (kit "A" or "B" and oligo number) and the differentially expressed band number on the gel.

Distinct classes of expression patterns were generated as discussed above.

The banding profile of individual transcripts may be grouped into distinct classes of expression patterns.

Class I:

The majority of transcripts belonged to class I, and were expressed by all cell types examined, as exemplified by *B073* and *B077* (Figure 4). These transcripts are therefore likely to represent housekeeping genes.

Class II:

A significant proportion of bands represented class II transcripts, expressed in EBM^0 and down regulated at various stages during differentiation, for example B096 (Figure 5) and A176 (Figure 6).

Class III:

Ten products, from five different primer combinations, were first expressed coincident with the formation of EPL cells, demonstrating up regulation on days 2 and 3. These products were classified as class III transcripts (Figure 7). Down regulation of class III transcripts varied between days 4 to 8 (Figure 7).

Class IV:

Nineteen transcripts, from all six primer combinations, exhibited banding profiles that potentially coincided with the formation of the postulated ectoderm cell population (Figure 8). These class IV transcripts displayed an up regulation in expression on days 4, 5 and 6. Although most transcripts were expressed up to day 9, three products were down regulated on day 7 when *Sox1* expression

indicated neurectoderm formation and were therefore potential markers for definitive ectoderm (Figure 8).

Class V:

Two other transcripts, classified as class V products, were identified as neurectoderm specific, exhibiting expression on days 8 and 9 only (data not shown).

Together, using the six random primer combinations, 54 differentially expressed fragments were identified during the entire ES to neurectoderm transition.

Expression Profiles of Potential Cell Markers

Class III and class IV products were selected for further analysis and are denoted by asterisks in Figure 7 and Figure 8. While up regulation of class III products at day 2 or 3 did not coincide with the putative definitive ectoderm, three class III products (A091, A193 and B078) were down regulated at day 7, providing a potential marker for late definitive ectoderm.

Class IV transcripts are predicted to be up regulated within ectoderm but not EPL cells and were segregated into three subclasses (Figure 8).

Subclass IV(i):

Products B072 (Figure 4), B094, B095, B099, B0910 (Figure 5), A175, A1712 (Figure 6) and A208 (not shown) comprised subclass IV(i) and were upregulated in EBM[4]. A1712, B072, B095 and A175 which were down regulated on days 7 and 9, were cloned from this series.

Subclass IV(ii):

Subclass IV(ii) consisted of mRNA transcripts up regulated at day 5. A191, A194 (Figure 9), B074 (Figure 4), A204 (not shown) and A1715 (Figure 6) were up

regulated at day 5 and remained expressed throughout the time course (Figure 9). A191 was cloned as a representative of this group. A1711 (Figure 6) was detected at low levels in EBM^5 to EBM^7 and was also cloned.

Subclass IV(iii):

The final subclass IV(iii) demonstrated up regulated expression at day 6 with continued expression until day 9. Of these, A205 (not shown), B098 (Figure 5) and A178 (Figure 6) were selected for further analysis.

As used herein, the term "neurectoderm" refers to undifferentiated neural progenitor cells substantially equivalent to cell populations comprising the neural plate and/or neural tube. Neurectoderm cells referred to herein retain the capacity to differentiate into all neural lineages, including neurons and glia of the central nervous system, and neural crest cells able to form all cell types of the peripheral nervous system.

The step of contacting the EPL cells with the conditioned medium may be conducted in any suitable manner. For example definitive ectoderm cells may be generated in adherent culture or as cell aggregates in suspension culture. Preferably the neurectoderm cells are produced in suspension culture in a culture medium such as DMEM, supplemented with the biologically active factor etc. Preferably the cells are cultured for approximately 1 to 7 days, more preferably approximately 4 to 6 days.

Again, a conditioned medium as hereinbefore described may be used to derive and maintain the definitive ectoderm cells or the conditioned medium may be fractionated to yield an extract therefrom exhibiting neural inducing properties, which may be added alone or in combination to other media to provide the neurectoderm cell deriving medium. The conditioned medium may be used undiluted or diluted (e.g. approx. 10-80%, preferably approximately 40-60%, more preferably approximately 50%).

In a further aspect of the invention there is provided a method for selectively producing neurectoderm cells or surface ectoderm cells from DEE cells, which method includes

providing

a source of DEE cells as hereinbefore described, and

a suitable culture medium;

a growth factor capable of promoting development of surface ectoderm; and

further culturing the DEE cells in the presence or absence of the growth factor, and optionally in the presence of additional growth factors and/or differentiation agents, to produce, in the presence of the growth factor, surface ectoderm cells, and in the absence of the growth factor, neurectoderm cells.

The developmental potential of definitive ectoderm during mouse embryogenesis has been studied by transplantation and fate mapping experiments (Beddington 1982; Gimlich and Cooke 1983; Hogan et al. 1994). These experiments demonstrate that definitive ectoderm gives rise to two subsequent embryonic tissue types which are more restricted in their developmental potential: the surface ectoderm and the neurectoderm. Whilst the surface ectoderm predominantly gives rise to such tissues as the epidermis, hair, lens and cornea which contribute to the skin and eye of the embryo respectively, cells of the neurectoderm develop into neurons and glia of the central nervous system (CNS) and sensory neurons, postganlglionic autonomic neurons and Schwann cells of the peripheral nervous system (PNS). Developmental bipotentiality to form these two ectodermal derivatives is distinctive of definitive ectoderm. Demonstration that the novel *in vitro* intermediate cell population described is capable of differentiation into both neurectoderm and surface ectoderm, but not mesoderm, supports the proposition that it represents a parallel definitive ectoderm population *in vitro*.

The method according to this aspect of the present invention is supportive of a 'default' model of neural induction recently proposed which states that neuralization of definitive ectoderm results from the inhibition of an epidermal

inductive signal (Weinstein and Hemmati-Brivanlou 1999).

The growth factor capable of inducing development of surface ectoderm may be a member of the transforming growth factor or epidermal growth factor families.

Members of the Transforming Growth Factor-β (TGF-β) gene family are good candidates for molecules involved in surface ectoderm induction. For example, Bone Morphogenetic Protein 4 (BMP4). Bone Morphogenetic Protein 2 (BMP2) or Activin may be used.

Similarly Epidermal Growth Factor (EGF) has been proposed to play an important role in epidermal development in the adult mouse by promoting proliferation and differentiation (keratinization) of the epidermis, and is required for the culture of epithelial cells *in vitro* (Partanen 1990; Sibilia and Wagner 1995).

Members of the epidermal growth factor family which may be used include epidermal growth factor (EGF), TGF-α and β-cellulin.

The effect of the growth factor, e.g. BMP4 on differentiation of the definitive ectoderm cell population *in vitro* may be ascertained by: a) morphological alterations in differentiated cells, b) relative effect on neuron formation, c) induction of surface ectoderm markers within the differentiated tissue and (d) down regulation of neurectodermal/neural markers within the differentiated tissue.

The concentration of the growth factor when present is preferably in the range approximately 1 to 100 ng/ml, more preferably approximately 5 to 50 ng/ml.

The concentration of the BMP4 growth factor may, for example, be of the order of 10 ng/ml. The concentration of the EGF growth factor may, for example, be of the order of 10 ng/ml. Optionally, both growth factors may be included, e.g. at similar concentrations.

For example, EBM^6, which were determined to be the most likely population

to represent definitive ectoderm, were cultured in the presence of the exogenous factors with aggregates and differentiated products examined morphologically until day 12. The relative formation of neurectoderm and surface ectoderm was assayed by expression of marker genes that distinguish these populations.

The neurectoderm cells so formed may in turn differentiate to form neuronal cells such as neurons and glia with high frequency.

The step of further culturing the DEE cells in the presence or absence of a growth factor and in the presence of additional growth factors and/or differentiation agents, may be conducted in any suitable manner. Preferably the cellular aggregates are removed from FCS and then cultured in the presence or absence of a growth factor in the presence of additional growth factors and/or differentiation agents, and in the absence of the conditioned medium.

For example, differentiated cells may be generated in adherent culture or as cell aggregates in adherent culture. Preferably the differentiated cells are produced in suspension culture in a suitable culture medium, e.g. containing insulin, transferrin and/or sodium selenite, such as DMEM: Ham's F12 nutrient mixture (F12), supplemented additional growth factors and/or differentiation agents and optionally with the growth factor. In a particularly preferred embodiment, the suitable culture medium may contain approximately 50:50 DMEM:F12. Preferably the cells are cultured for approximately a further 1 to 10 days, more preferably approximately 1 to 5 days.

Accordingly, in a further aspect of the present invention, there is provided a surface ectoderm cell derived *in vitro* and characterised by two or more of

early epidermal-like morphology;
expression of Keratin 18 (K18); and/or
expression of Keratin 14 (K14);
substantial absence of expression of Sox1 and nestin.

In a further aspect of the present invention there is provided a method for producing genetically modified definitive ectoderm equivalent (DEE) cells which

method includes

providing

a source of early primitive ectoderm-like (EPL) cells; and

a conditioned medium as hereinbefore defined; or an extract therefrom exhibiting neural inducing properties; and

culturing the EPL cells in the conditioned medium or extract for a time sufficient to permit controlled differentiation to DEE cells; and

modifying one or more genes in the DEE cells; or

modifying one or more genes in the EPL cells prior to culturing in conditioned medium or extract, to produce generitally modified definitive ectoderm equivalent (DEE) cells.

Modification of the genes of these cells may be conducted by any means known to the skilled person which includes introducing extraneous DNA, removing DNA or causing mutations within the DNA of these cells. Modification of the genes includes any changes to the genetic make-up of the cell thereby resulting in a cell genetically different to the original cell.

The genetically modified or unmodified definitive ectoderm cells of the present invention and the differentiated or partially differentiated cells derived therefrom, include neurectoderm and surface ectoderm, may be generated in amounts that allow widespread availability for therapeutic and commercial uses. The cells have a number of uses, including the following:

- use in human cell therapy to treat and cure neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, and other pathological conditions including stroke and spinal cord injury. For example ectoderm cells or their differentiated or partially differentiated progeny produced from DEE cells may be used to replace or assist the normal function of diseased or damaged tissue. For example in Parkinson's disease the dopaminergic cells of the substantia nigra are progressively lost. The dopaminergic cells in Parkinson's patients could be replaced by implantation of neural

cells, produced from ectoderm in the manner described in this application.

Similarly, precursors of adult skin, hairs, lens and cornea of the eye, including surface ectoderm and its derivatives may be used for transplantation therapy. A number of corneal disorders may be treated by corneal transplant, including corneal clouding, degeneration following cataract surgery, keratoconus, bullous keratopathy and chemical burns. Currently corneas for transplant are sourced from deceased donors. Similarly skin grafts are used to treat a number of conditions, most notably burns.

Surface ectoderm derived *in vitro* may be further differentiated into a number of surface tissues including corneal epithelia, skin and lens, providing an alternative source in potentially unlimited amounts of those tissues for transplant.

- use to produce cells, tissues or components of organs for transplant. For example, surface ectoderm may be induced to produce skin cells, lens or corneal cells of the eye and hair which potentially may be used for transplants.

- use in human gene therapy to treat neuronal, skin, dye or hair diseases. In one approach neurectoderm cells or their differentiated and partially differentiated products may be genetically modified so that they provide functional biological molecules. The genetically modified cells can be implanted, thus allowing appropriate delivery of therapeutically active molecules.

- use as a source of cells for reprogramming. For example karyoplasts from neurectoderm or definitive ectoderm or their differentiated or partially differentiated progeny may be reprogrammed by nuclear transfer. Cytoplasts from neural cells may also be used as vehicles for reprogramming so that nuclear material

derived from other cell types are directed along neural and ectodermal lineages. Alternatively neural stem cells may be reprogrammed in response to environmental and biological signals that they are not normally exposed to. Hence neurectoderm and ectoderm cells described herein are potentially capable of forming differentiated cells of non-neural or non-ectoderm lineages, including cells of mesodermal lineage, such as haematopoietic cells and muscle. Reprogramming technology using neural or ectodermal cells potentially offers a range of approaches to derive cells for autologous transplant. In one approach karyoplasts from differentiated cells are obtained from the patient, and reprogrammed in neurectoderm or ectoderm cytoplasts to generate autologous neurectoderm. The autologous neurectoderm or ectoderm cells or their differentiated or partially differentiated progeny could then be used in cell therapy to treat neurodegenerative diseases.

- use in pharmaceutical or toxicological screening for therapeutic drugs that influence the behaviour of neurectoderm or definitive ectoderm cells and their differentiated or partially differentiated progeny.

- use in the evaluation of biological molecules that direct differentiation of definitive ectoderm cells.

In a still further aspect of the present invention, there is provided a method for the preparation of tissue or organs for transplant, which method includes

providing surface ectoderm cells produced as described above;

culturing the surface ectoderm cells to produce skin cells, lens or corneal cells of the eye, or hair cells; and

transplanting the cells produced to a selected site within the patient's body.

The present invention will now be more fully described with reference to the accompanying examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as

a restriction on the generality of the invention described above.

In the figures:

Figure 1

Changes in the morphology of Embryoid Bodies cultured in the presence of MEDII (EBMs) over nine days

ES cells were aggregated in suspension in incomplete ES cell media supplemented with 50% MEDII and cultured for seven days. On day 7, EBMs were transferred to 50% DMEM: 50% Hams F12 supplemented with ITSS and 10 ng/ml FGF2 and cultured for a further two days.

A EBM^2

At 2 days of culture, cellular aggregates formed in the presence of MEDII were perceived as a solid ball of cells with individual cells easily discernible.

B EBM^4

By day 4 of culture, cells had proliferated rapidly such that the solid mass of cells had enlarged in size and individual cells were no longer discernible.

C EBM^7

By day 7, the ball of cells had converted into an epithelial sheet of cells and comprised a convoluted cell layer.

D EBM^9

At day 9, the cell layer appeared to thicken and organise into a stratified epithelial sheet of columnar cells.

Arrows indicate the convoluted epithelial layer.

Figure 2

Analysis of gene expression during differentiation of pluripotent cells as EBM

A. 20 μg of total RNA from ES cells and EBM^{1-9} was analysed by Northern blot for the expression of *Rex1*, *brachyury* and *mGAP*. *Rex1* transcripts were 1.9 kb (Hosler et al., 1989), *brachyury* 2.1 kb (Lake et al., 2000) and *mGAP* 1.5 kb. **B.** 20 μg RNA from EBM^{4-8} was analysed for the expression of *Oct4* and *mGAP* by Northern blot analysis. *Oct4* transcripts were 1.55 kb (Rosner et al., 1990) and *mGAP* 1.5 kb. **C.** RNase protection analysis of 10 μg total RNA from EBM^{4-9} with radiolabelled antisense probes for *Sox1* and *mGAP*. Protected bands are indicated in the figure. **D.** The level of expression of *Oct4* and *Sox1* relative to mGAP expression was determined from Northern analysis (B) and RNase protection (C) respectively and graphed. Band intensities were measured using ImageQuant software.

Figure 3

Temporal description of differentiation events and correlation with events *in vivo* by gene expression analysis.

A: In the embryo, fertilisation of the oocyte is denoted by 0 dpc By 4.5 dpc the inner cell mass (ICM) has formed indicated by the up regulation of *Rex1* and *Oct-4. Rex1* expression is decreased and by 4.75 dpc is no longer detected. This coincides with primitive ectoderm formation. *Oct-4* is still expressed but levels are lower. Formation of the neurectoderm at 7.5 dpc is demonstrated by up regulation of *Sox1*.

B: The neuronal differentiation system in which ES were cultured as embryoid body aggregates in the presence of MEDII for nine days. Day 0 represents undifferentiated ES cells, demonstrated by the expression of *Rex1* and *Oct-4* and correlated with the ICM *in vivo*. The down regulation of *Rex1* at day 2 indicates the formation of EPL cells, which correlates with the beginning of

primitive ectoderm formation *in vivo.* Neurectoderm is present at day 7, indicated by up regulation of *Sox1* and corresponds to 7.5 dpc in the embryo.

Figure 4

ddPCR Profile of EBM^{0-9} using the OPB-07 5' Arbitrary Oligonucleotide

5 μg total RNA was reverse transcribed with 3'-anchored oligo dT_{12} primer for 50 min at 42°C in a 20 μl volume. $1/10^{th}$ of undiluted first strand cDNA was used as the template for ddPCR with OPB-07. [α-^{33}P] dATP was incorporated. ddPCR products were labelled by the arbitrary primer and differentially expressed band number on the gel. EBM^{0-9} ddPCR reactions were run on the same gel. Sizes are indicated.

Examples of differentially expressed genes:

Class I:	B073, B077
Class III:	B078
Class IV:	B072, B074

Figure 5

ddPCR Profile of EBM^{0-9} using the OPB-09 5' Arbitrary Oligonucleotide

5 μg total RNA was reverse transcribed with 3'-anchored oligo dT_{12} primer for 50 min at 42°C in a 20 μl volume. $1/10^{th}$ of undiluted first strand cDNA was used as the template for ddPCR with OPB-09. [α-^{33}P] dATP was incorporated. ddPCR products were labelled by the arbitrary primer and differentially expressed band number on the gel. EBM^{0-9} ddPCR reactions were run on the same gel. Sizes are indicated.

Examples of differentially expressed genes:

Class II:	B096

Class IV: B092, B094, B095, B098, B099, B0910

Figure 6

ddPCR Profile of EBM^{0-9} using the OPA-17 5' Arbitrary Oligonucleotide

5 μg total RNA was reverse transcribed with 3'-anchored oligo dT_{12} primer for 50 min at 42°C in a 20 μl volume. 1/10th of undiluted first strand cDNA was used as the template for ddPCR with OPA-17. [α-^{33}P] dATP was incorporated. ddPCR products were labelled by the arbitrary primer and differentially expressed band number on the gel. EBM^{0-9} ddPCR reactions were run on the same gel. Sizes are indicated.

Examples of differentially expressed genes:

Class II: A176

Class IV: A175, A178, A1711, A1712

Figure 7

Summary of Potential Class III Cell Markers

Expression patterns of potential class III differentially expressed products, summarised from figures 4.1, 4.2 and 4.3 and all other ddPCR gels (data not shown). Three of these products were selected for reamplification and cloning, and are denoted by asterisks. The sizes of each of these products are shown.

Figure 8

Summary of Potential Class IV Cell Markers

Expression patterns of potential Class IV differentially expressed products, summarised from figures 4.1, 4.2 and 4.3 and all other ddPCR gels (data not shown). Nine of these products were selected for reamplification and cloning, and

are denoted by asterisks. The sizes of each of these products are shown.

Figure 9

ddPCR Profile of EBM0-9 using the OPA-19 5' Arbitrary Oligonucleotide

5 μg total RNA was reverse transcribed with 3'-anchored oligo dT_{12} primer for 50 min at 42°C in a 20 μl volume. $1/10^{th}$ of undiluted first strand cDNA was used as the template for ddPCR with OPA-19. [α-^{33}P] dATP was incorporated. ddPCR products were labelled by the arbitrary primer and differentially expressed band number on the gel. EBM^{0-9} ddPCR reactions were run on the same gel. Sizes are indicated.

Examples of differentially expressed genes:

Class III:	A193
Class IV:	A191, A194

Figure 10

Reamplification of ddPCR Products

A193, B078, A175, A1712, B098 and A205 ddPCR products were excised, eluted and reamplified by PCR using the original oligonucleotides (2.3.14). 1 μl of the 25 μl PCR reaction was run on a 2% TAE agarose gel and the sizes of reamplified fragments determined by pUC marker DNA.

Figure 11

Detection and Localisation of A175, B072, A17113A and A1712 Expression in EBM^6 and EBM^9

Detection and localisation of A175, B072, A17113A and A1712 expression in embryoid bodies cultured in the presence of MEDII at day 6 (EBM^6) and day 9

(EBM^9) was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes of the cDNA clones. Cells that expressed A175, B072, A17113A or A1712 were indicated by pink staining. Photographs were taken under Hoffman optics at 200 X magnification.

A/B Detection of A175 transcript in EBM^6 (A) and EBM^9 (B)

C/D Detection of B072 transcript in EBM^6 (C) and EBM^9 (D)

E/F Detection of A17113A transcript in EBM^6 (E) and EBM^9 (F)

G/H Detection of A1712 transcript in EBM^6 (G) and EBM^9 (H)

Figure 12

The effect of BMP4 on neuron formation from EBMs

EBM6 were transferred to gelatinised wells and replaced with 50% F12: 50% DMEM supplemented with 1/1000 ITSS and 1/1000 glutamine. 10 ng/ml BMP4 was added and the bodies cultured until day 12. Neuron differentiation was assessed morphologically by the presence of neurons within individual embryoid bodies. Photographs were taken under Hoffmann optics at 200 X magnification.

A: Morphology of EBMs on day 12 of differentiation. Bundles of axons were visible emanating from individual bodies.

B: Morphology of EBMs cultured in the presence of 10 ng/ml BMP4 on day 12 of differentiation. Bundles of axons could not be seen in most bodies.

C: Neurons were visible in 87.1% of EBMs cultured in the absence of BMP4. The addition of 10 ng/ml BMP4 in the culture media significantly reduced ($p<0.05$) neuronal differentiation such that only 18.1% of the 44 bodies examined exhibited any morphological sign of neuron formation. Statistical significance was determined by analysis using Students unpaired T-test.

Figure 13

Morphology changes of EBMs and differentiated cells upon addition of BMP4

A/B EBM cultured without BMP4.

Photographs were taken on day 8 under Phase Contrast optics at (A) 100 X and (B) 200 X magnification.

C/D EBM cultured in the presence of 10 ng/ml BMP4.

Photographs were taken on day 8 under Phase Contrast optics at (A) 100 X and (B) 200 X magnification.

Figure 14

Detection and Localisation of Sox1 Expression in EBM^9

Detection and localisation of *Sox1* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *Sox1* cDNA plasmid. Cells that expressed *Sox1* were indicated by purple staining. Photographs were taken under Phase Contrast optics at 100 X magnification.

A EBM cultured without BMP4.
B EBM cultured with 10 ng/ml BMP4.

Figure 15

Detection and Localisation of nestin Expression in EBM^8 and EBM^9

Detection and localisation of *nestin* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript

expression was detected using DIG labelled antisense riboprobes generated from the *nestin* cDNA plasmid. Cells that expressed *nestin* were indicated by pink staining. Photographs were taken under Phase Contrast optics at 100 X magnification.

A EBM^8 cultured without BMP4.
B EBM^8 cultured with 10 ng/ml BMP4.
C EBM^9 cultured with 10 ng/ml BMP4.

Figure 16

Detection and Localisation of *K18* Expression in EBM^9

Detection and localisation of *K18* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *K18* cDNA plasmid. Cells that expressed *K18* were indicated by purple staining. Photographs were taken under Phase Contrast optics at 100 X and 200 X magnification.

A EBM^9 cultured without BMP4. 100 X magnification.
B EBM^9 cultured with 10 ng/ml BMP4. 100 X magnification.
C EBM^9 cultured with 10 ng/ml BMP4. 200 X magnification.

Figure 17

Detection and Localisation of *K14* Expression in EBM^8

Detection and localisation of *K14* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *K14* cDNA plasmid. Cells that expressed *K14* were indicated by purple staining. Photographs were taken under Brightfield optics at 200 X magnification.

A EBM^8 cultured without BMP4.

B EBM^8 cultured with 10 ng/ml BMP4.

Figure 18

Detection and Localisation of *brachyury* Expression in EBM^9

Detection and localisation of *brachyury* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *brachyury* cDNA plasmid. Photographs were taken under Phase Contrast optics at 200 X magnification.

A EBM^9 cultured without BMP4.

B EBM^9 cultured with 10 ng/ml BMP4.

Figure 19

Detection and Localisation of *Sox1* Expression in EBM^8

Detection and localisation of *Sox1* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 and/or 10 ng/ml EGF was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *Sox1* cDNA plasmid. Cells that expressed *Sox1* were indicated by pink staining. Photographs were taken under Phase Contrast optics at 100 X and 200 X magnification.

A/B EBM^8 at (A) 100 X magnification and (B) 200 X magnification.

C/D EBM^8 cultured with 10 ng/ml BMP4 at (A) 100 X magnification and (B) 200 X magnification.

E/F EBM^8 cultured with 10 ng/ml EGF at (A) 100 X magnification and (B) 200 X magnification.

G/H EBM^8 cultured with 10 ng/ml BMP4 + 10 ng/ml EGF at (G) 100 X magnification and (H) 200 X magnification.

Figure 20

Detection and Localisation of *nestin* Expression in EBM[8]

Detection and localisation of *nestin* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 and/or 10 ng/ml EGF was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *nestin* cDNA plasmid. Cells that expressed *nestin* were indicated by pink staining. Photographs were taken under Phase Contrast optics at 100 X magnification.

A EBM[8].
B EBM[8] cultured with 10 ng/ml BMP4.
C EBM[8] cultured with 10 ng/ml EGF.
D EBM[8] cultured with 10 ng/ml BMP4 + 10 ng/ml EGF.

Figure 21

Detection and Localisation of *K18* Expression in EBM[8]

Detection and localisation of *K18* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 and/or 10 ng/ml EGF was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *K18* cDNA plasmid. Cells that expressed *K18* were indicated by purple staining. Photographs were taken under Phase Contrast optics at 100 X and 200 X magnification.

A/B EBM[8] at (A) 100 X magnification and (B) 200 X magnification.

C/D EBM[8] cultured with 10 ng/ml BMP4 at (A) 100 X magnification and (B) 200 X magnification.

E/F EBM[8] cultured with 10 ng/ml EGF at (A) 100 X magnification and (B) 200 X magnification.

G/H EBM[8] cultured with 10 ng/ml BMP4 + 10 ng/ml EGF at (G) 100 X magnification and (H) 200 X magnification.

Figure 22

Detection and Localisation of *K14* Expression in EBM^8

Detection and localisation of *K14* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *K14* cDNA plasmid. Cells that expressed *K14* were indicated by purple staining. Photographs were taken under Brightfield optics at 100 X, 200 X and 400 X magnification.

A/B EBM^8 at (A) 100 X magnification and (B) 200 X magnification.

C/D/E EBM^8 cultured with 10 ng/ml EGF at (C) 100 X magnification and (D) 200 X magnification and (E) 400 X magnification.

F/G EBM^8 cultured with 10 ng/ml BMP4 + 10 ng/ml EGF at (E) 100 X magnification and (F) 200 X magnification.

Figure 23

Detection and Localisation of *brachyury* Expression in EBM^8

Detection and localisation of *brachyury* expression in embryoid bodies cultured with and without 10 ng/ml BMP4 and/or 10 ng/ml EGF was carried out by *in situ* hybridisation. Transcript expression was detected using DIG labelled antisense riboprobes generated from the *brachyury* cDNA plasmid. Photographs were taken under Phase Contrast optics at 100 X magnification.

A EBM^8.

B EBM^8 cultured with 10 ng/ml BMP4.

C EBM^8 cultured with 10 ng/ml EGF.

D EBM^8 cultured with 10 ng/ml BMP4 + 10 ng/ml EGF.

EXAMPLES

Materials and methods

Abbreviations

APS	ammonium persulphate
ATP	adenosine triphosphate
β-ME	β-Mercaptoethanol
bp	base pair
BCIG	5-bromo-4-chloro-3-indolyl-beta-D-galactosidase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
cDNA	complementary DNA
c.p.m.	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTPs	deoxyribonuceotide triphosphates
DIG	digoxygenin
DMEM	Dulbecco's Modified Eagles Medium
DMF	dimethyl formamide
DMSO	dimethylsulphoxide
dpc	days post-coitum
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
EPL	early primitive ectoderm-like
ES	embryonic stem
EtBr	ethidium bromide
EtOH	ethanol
FCS	foetal calf serum

GLB	gel loading buffer
ITSS	Insulin-transferrin-sodium-selenite
HEPES	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
min	minute
mLIF	mouse leukaemia inhibitory factor
MOPS	3-[N-morpholino]propane sulphonic acid
MQ	milli-Q
NBT	4-nitroblue tetrazolium chloride
O/N	overnight
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
RNase	ribonuclease
RNAsin	ribonuclease inhibitor
rNTPs	ribonucleotide phosphates
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
TAE	tris acetate EDTA
TBE	tris borate EDTA
TEMED	N,N,N',N'-tetramethyl-ethenediamine
tRNA	transfer RNA
Tween-20	polyoxyethylene-sorbitan Monolaurate
U	unit(s)
UV	ultra violet
V	volts

Materials

Chemicals and Reagents

Sigma Chemical Co. supplied the following chemicals: Agarose (Type 1),

APS, β-ME, Brilliant Blue (Coomassie), Bromophenol Blue, BSA, DMSO, DTT, EtBr, EDTA, gelatin, mineral oil, MOPS, rNTPs, SDS, TEMED, Tween 20.

Sources for other important reagents were as follows: Acrylamide; GeneWorks. IPTG, BCIG; Progen. Anti-DIG Fab antibody-alkaline phosphatase conjugate, BCIP, glycogen, ITSS, NBT and tRNA; Roche Molecular Biochemicals. Ammonium acetate, APS, chloroform, formaldehyde, formamide, methanol, magnesium acetate, NP-40, PEG 6000 and phenol, Tris-HCl, Triton X-100; BDH Chemicals. Isopropanol; Ajax Chemicals. Paraformaldehyde; Merck. Blue Dextran, Poly dT_{12-18} primer, Sephadex G-50 fine and Sepharose CL-6B; Pharmacia. Gluteraldehyde; Probing and Structure. Dithioerythritol and DTT; Diagnostic Chemicals Ltd. Sequagel 6; National Diagnostics.

All chemicals and reagents were of analytical grade.

Radiochemicals

[α-^{32}P] dATP (3000 Ci/mmol), [a-^{33}P] dATP (3000 Ci/mmol), [a-^{32}P] dUTP (3000 Ci/mmol) were supplied by GeneWorks.

Kits

Bresaclean kit	GeneWorks
Gigaprime kit	GeneWorks
Midiprep kit	Bio Rad
RNAzol B kit	Ambion

Enzymes

Restriction endonucleases were supplied by Pharmacia and New England Biolabs. Other enzymes were obtained from the following sources: Proteinase K; Boehringer Mannheim. Klenow DNA polymerase I, Taq polymerase, RNasin, DNase I, T4 DNA ligase, SP6 RNA polymerase, T7 RNA polymerase and T3 RNA polymerase; GeneWorks. RNase A; Sigma. RNase T1; Ambion. T7 DNA

polymerase; Pharmacia. Superscript II reverse transcriptase; Gibco BRL.

All enzymes were stored to the manufacturers specifications.

Buffers and Solutions

Buffer 1:	100 mM Tris-HCl pH 7.4, 100 mM NaCl
Buffer 2:	Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$
Buffer 3:	Buffer 1, 1 mM EDTA.
FLB:	Deionised formamide, 0.02% bromophenol blue, 0.02% xylene cyanol
Fragment Elution Buffer:	0.5 M NH_4Ac, 10 mM $MgAc_2$, 1 mM EDTA pH 8.0, 0.1% SDS
GET buffer:	25 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose
GLB:	50% glycerol, 0.1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol
β-ME/PBS:	100 mM β-ME (Sigma) in 14 ml PBS. Fresh solution prepared fortnightly
MOPS:	23 mM MOPS pH 7.0, 50 mM NaAc, 10 mM EDTA pH 8.0
PBS:	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 pH 7.4 Sterilised by autoclaving (20 psi for 25 min at 140°C)
PBS/Gelatin:	0.2% (w/v) gelatin in PBS. Sterilised by autoclaving (20 psi for 25 min at 140°C)
RIPA buffer:	150 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8.0
SD buffer:	33 mM Tris-HAc pH 7.8, 62.5 mM KAc, 10 mM MgAc, 4 mM spermidine, 0.5 mM dithioerythritol
SSC:	150 mM NaCl, 15 mM sodium citrate pH 7.4
Ste buffer:	150 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA
TAE:	40 mM Tris-acetate, 20 mM NaAc, 1 mM Na_2EDTA pH 8.2
TBE:	90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA pH 8.3
Trypsin:	0.1% trypsin (Difco) and 1 x EDTA Versene buffer solution (CSL) Sterilised by filtration through a 0.2 mm filter

(Whatmann)

Plasmid Vectors

Cloned DNA sequences

The *brachyury* cDNA clone was provided by Dr B. Herrmann. The plasmid contained a 1764 bp of *brachyury* cDNA cloned into the *Eco*RI site of Bluescript II SK (Herrmann 1991).

The *mGAP* (mouse Gluteraldehyde Phosphate Dehydrogenase) cDNA clone in pGEM3Z was from Prof. P. Rathjen and contained a 300 bp *Hind*III/*Pst*I fragment from the 5' end of mouse *GAPDH* gene (Rathjen et al. 1990b).

The *Oct-4* cDNA clone in pBluescript was provided by Dr H. Schöler and contained a 462bp *Stu*I cDNA fragment of positions 491 to 953 of the *Oct-4* cDNA sequence (Schöler et al., 1990b).

Rex1 was donated by Dr N. Clarke and contained 848 bp of *Rex1* cDNA in the *Eco*RI site of pCRTMII (Hosler et al. 1989).

Sox1 was obtained from Dr R. Lovell-Badge and contained 1.1 kb of the *Sox1* sequence in Bluescript KS. A 450 bp *RI*/*Xho*I fragment was subcloned into Bluescript KSII (J.Rathjen; unpublished data).

The *K18* cDNA clone was donated by Dr R. Oshima (Singer et al., 1986) and contained a 1163 bp fragment cloned into the *Hind*III site of pT7/T319U.

The *K14* clone was obtained from Prof. G. Rogers and contained a 450 bp fragment of the 3' non-coding region of the mouse *K14* gene cloned into pGEM3.

Riboprobe Templates

Radioactive riboprobes were synthesised as described below. The generation of DIG labelled riboprobes is described below.

Oct-4

Riboprobes for *in situ* hybridisation were generated from the *Oct-4* cDNA plasmid. The antisense transcript was generated by digestion with *Hind*III and transcription with T7 polymerase. The sense transcript was generated by restriction with *Xho*I and transcription with T7 polymerase.

Sox-1

Riboprobes for *in situ* hybridisation were generated from the *Sox-1* cDNA plasmid containing the 1.1 kb *Sox1* sequence. The antisense transcript was generated by digestion with *BamHI* and transcription with T3 polymerase. The sense transcript was generated by restriction with *Hind*III and transcribed with T7 polymerase.

Riboprobes for ribonuclease protection were obtained from the *Sox-1* cDNA plasmid containing the 450 bp *RI/Xho*I fragment. The antisense transcript was generated by digestion with *Kpn*I and transcription with T7 polymerase. The sense transcript was generated by restriction with *Pst*I and transcription with T3 polymerase.

Brachyury

Riboprobes for Northern blot and *in situ* hybridisation were generated from the *brachyury* plasmid. The antisense transcript was generated by digestion with *Bam*HI and transcription with T7 polymerase. The sense transcript was obtained by restriction with *Sal*I and transcription with T3 polymerase.

K18

Riboprobes for *in situ* hybridisation were generated from the *K18* plasmid. The antisense transcript was generated by digestion with *Eco*RI and transcription with T7 polymerase. The sense transcript was generated by restriction with *Kpn*I

and transcribed with T3 polymerase.

K14

Riboprobes for *in situ* hybridisation were generated from the *K14* plasmid. The antisense transcript was generated by digestion with *Eco*RI and transcription with sp6 polymerase. The sense transcript was generated by restriction with *Hind*III and transcribed with T7 polymerase.

Nestin

Riboprobes for *in situ* hybridisation and northern blot were generated from the *N500* plasmid. The antisense transcript was generated by *Nco*I digestion and transcription with sp6 polymerase. The sense transcript was generated by digestion with *Sal*I and transcription with T7 RNA polymerase.

mGAP

Riboprobes for ribonuclease protection were generated from the *mGAP* cDNA plasmid. The plasmid was linearised with *Pst*I and transcribed with T3 polymerase.

Oligonucleotides

DNA primers were synthesised by GeneWorks. 5' arbitrary ddPCR primers, OPA and OPB 10mer kits were purchased from Operon technologies. Primer sequences are shown 5'-3'. *Eco*RI restriction sites are in bold.

General sequencing primers

T7:	TAATACGACTCACTATAGGGAGA
T3:	ATTAACCCTCACTAAAGGGA

ddPCR primers

Anchored 3' oligo dT_{12} primer:

AT**GAATTC**TTTTTTTTTTTTA

Arbitrary 5' primers:

OPA-09:	GGGTAACGCC	OPA-20:	GTTGCGATCC
OPA-17:	GACCGCTTGT	OPB-07:	GGTGACGCAG
OPA-19:	CAAACGTCGG	OPB-09:	TGGGGGACTC

RT-PCR primers:

Nestin 501: GGAGGGCAGAGAAGACAGTG
Nestin 502: TGACATCCTGGACCTTGACA

Bacterial Strains

All recombinant plasmids were maintained in *E.coli* DH5α. O/N cultures diluted with an equal volume of 80% glycerol were used for long term storage of stock strains and transformed bacteria at -80°C.

DH5α: *sup*E44 Δ*lac* U169 (phi80*lac*ZdeltaM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

Bacterial Growth Media

Growth media were prepared in milliQ filtered water and sterilised by autoclaving. Antibiotics and other labile chemicals were added after the media solution had cooled to 50°C.

Luria broth (LB): 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, pH 7.0 (adjusted with NaOH).

Psi broth: 2% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) $MgSO_4$, pH 7.6 (adjusted with 1 M KOH).

Solid Media: Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar (Difco).

Ampicillin (100 mg/ml) (Sigma Chemical Co.) was added where appropriate for growth of transformed bacteria to maintain selective pressure for recombinant plasmids.

Tissue Culture Cell Lines and Media

ES Cell Lines

The ES cell lines used throughout the course of this work were D3 and were derived from the ICM of the preimplantation 129 strain mouse embryo blastocyst (Doetschmann et al. 1985; Kindly donated by Dr L. Williams, Ludwig Institute, Melbourne, Australia).

Other Cell Lines

The Hep G2 cell line was derived from a human hepatoblastoma primary tumour (Knowles et al. 1980; ATCC HB-8065).

Media

Complete ES cell media: DMEM (Gibco BRL), pH 7.4, containing glucose; 10% FCS (Commonwealth Serum Laboratories), 1 mM L-glutamine, 0.1 mM β-ME/PBS, 1000 units of LIF (Rathjen et al. 1999) under 10% CO_2 in a humidified incubator.

Incomplete ES cell media: ES complete media without LIF.

MEDII conditioned medium: medium was isolated from Hep G2 cells cultured in incomplete ES cell medium for 4 days (Rathjen et al. 1999).

All tissue culture media were filter sterilised.

DNA Markers

*Hpa*II digested pUC19 markers were purchased from GeneWorks. Band sizes (bp): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26.

*Eco*RI digested SPP-1 phage markers were purchased from GeneWorks. Band sizes (kb): 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.

Miscellaneous Materials

3M chromatography paper	Whatmann Ltd.
Bio-max MR imaging film	Kodak
Hybond-XL nylon membrane	Amersham Pharmacia
Petri-dishes, 6-, 24-, 96-well trays	Falcon

Molecular Methods

Restriction Endonuclease Digestion of DNA

Plasmid DNA was digested in SD buffer with 1-2 U of enzyme/μg DNA and incubation at the appropriate temperature for 30 min to 3 hours. Complete digestion of DNA was assayed by agarose gel electrophoresis (Sambrook et al. 1989).

Agarose Gel Electrophoresis

Agarose gel electrophoresis (0.8% to 3% w/v agarose in TAE for DNA or TBE for RNA) was carried out using horizontal mini-gels prepared by pouring 10 ml of gel solution onto a 7.5 cm x 5.0 cm glass microscope slide. Agarose mini-gels were submerged in 1 x TAE or 1 x TBE and samples containing 1 x GLB were electrophoresed at 100 mA. Nucleic acid was visualised by staining gels with

EtBr (4 mg/ml in water) and exposure to medium wavelength UV light. Appropriate bands were removed from preparative gels using a sterile scalpel blade (Sambrook et al. 1989).

Polyacrylamide Gel Electrophoresis

Denaturing gels

6% polyacrylamide sequencing gels (20 cm x 40 cm x 0.37 mm, or 35 cm x 40 cm x 0.37 mm) were prepared from Sequagel 6 solutions according to the manufacturer's instructions. Prior to pouring the gel, 1/100 10% APS and 1/8000 TEMED were added to the gel solution to initiate polymerisation. Once the gel had set (60-90 min), the comb was removed and the wells flushed with water. Denaturing gels were pre-electrophoresed for 60 min at 45 W (small gel) or 90 min at 60 W (large gel) in order to heat the gel to 50°C. The wells were flushed with 1 x TBE before loading samples and electrophoresis was carried out at the same setting (approximately 50°C). After the gel had run, the glass plates were prised apart and the gel transferred to dry 3MM Whatmann paper. The gel was dried at 65°C under vacuum. Radioactivity was detected by exposure to X-ray film at RT.

Purification of Linear DNA Fragments

Linear DNA fragments were purified from agarose gels using the Bresaclean kit according to manufacturer's instructions. Briefly, the DNA band of interest was excised from an agarose gel and the agarose dissolved. The DNA was bound to a uniform size silica matrix under high salt conditions and then recovered in water.

Preparation of Vector DNA

Cloned nestin Sequence N500:

20mer primers (Nestin 501 and Nestin 502) were designed against a 500 bp (N500) fragment of the mouse *nestin* gene corresponding to positions

4553 to 5052. The fragment was amplified from 10.5 dpc mouse embryo cDNA (kindly provided by Stephen Rodda) using the RT-PCR method. The amplified product was purified by agarose gel electrophoresis and Bresaclean, then ligated into pGEM-T easy. The orientation of the insert was determined by digestion with *Sca*I and *Pst*I restriction enzymes.

ddPCR Cloning Vectors:

5-10 mg PCR DNA was purified by agarose gel electrophoresis and Bresaclean and ligated into pGEM-T easy (Promega).

DNA Ligation Reactions

Ligation reactions contained 50 ng purified vector, 3 mg purified PCR product, ligation buffer (60 mM Tris pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol) and 1 U T4 DNA ligase, and were incubated at RT for 1 hour.

Preparation of Competent Cells

A single *E.coli* colony was used to inoculate 5 ml Psi broth and grown in an orbital shaker O/N at 37°C. A 1:30 subculture was made in 15 ml Psi broth and grown for 90 min at 37°C, or until O.D. 0.6 at λ_{600}nm was obtained. A 1:20 subculture was made in 100 ml Psi broth and grown at 37°C until the O.D. was 0.5-0.6 at λ_{600}nm. The bacterial cells were chilled on ice for 5 min and harvested by centrifugation at 6000 rpm for 5 min at 4°C. The cells were resuspended in 0.4 volume of Tfb 1 and chilled on ice for a further 5 min. The cells were re-harvested by centrifugation and resuspended in a 0.04 volume of Tfb 2. After incubation on ice for 15 min. 50µl aliquots of cell suspension were transferred to eppendorf tubes and snap frozen in a dry ice/ethanol bath. The cells were stored at -80°C until required.

Transformation of Competent Cells

Approximately 10 ng of ligated DNA was added to 50µl of thawed competent cells and placed on ice for 30 min. The cells were heat shocked at 42°C for 90 sec and incubated in 800 ml of LB at 37°C for 30 min. The cells were pelleted by centrifugation and the supernatant decanted. The cells were resuspended in the residual supernatant, spread onto LB ampicillin (100 µg/ml) plates and transformant colonies grown O/N at 37°C. Where blue/white colour selection was required, plates were prepared by spreading with 105 µl IPTG (100 mM) and 20 µl BCIG.

Rapid Small Scale Preparation of DNA (Mini-prep)

2 ml LB broth ampicillin (100 µg/ml) was inoculated with a single transformant colony and incubated O/N at 37°C with shaking. 1.5 ml of cell cultures were transferred to eppendorf tubes and centrifuged for 5 min. The bacterial pellets were resuspended in 100 µl GET buffer and incubated at RT for 5 min. The bacteria were lysed by addition of 200 µl 0.2 M NaOH/1% SDS and chromosomal DNA precipitated by addition of 3 M NaAc (pH 4.6) followed by incubation at 4°C for 20 min. Samples were centrifuged for 15 min and supernatant collected and phenol/choloroform extracted to remove bacterial proteins. Plasmid DNA was precipitated by addition of isopropanol and incubated at -20°C for 10 min. Plasmid DNA was pelleted by centrifugation for 15 min and resuspended in 50 µl TE buffer containing 1:500 RNase A (10 mg/ml).

Rapid Large Scale Preparation of DNA (50 ml Midi-prep)

50 ml L broth ampicillin (100 µg/ml) was inoculated with a single transformant colony and incubated O/N at 37°C with shaking. Plasmid DNA was extracted using the Midiprep kit according to manufacturer's instructions. Briefly, bacterial cells were alkaline lysed and the plasmid DNA bound to a silica matrix and then recovered in water. Plasmid DNA was stored at -20°C.

Double Stranded Sequencing of Plasmid DNA

1 μg plasmid DNA was subjected to PCR based cycle sequencing in the presence of 100 ng USP or RSP primer and 8 μl Dye terminator ready reaction mix (Perkin Elmer) in a total volume of 10 ml. The reactions were amplified in a MJ Research PTC-100 thermal cycler for 25 cycles of: (1) 96°C for 10 sec; (2) 50°C for 5 sec; (3) 60°C for 4 min and the DNA precipitated in 1/10 volume 3 M NaAc, pH 4.6 and 2.5 volume 100% ethanol for 30 min at -80°C. Precipitated DNA was pelleted by centrifugation at 14,000 rpm for 20 min, washed in 70% ethanol, and dried by vacuum centrifugation. Reactions were analysed at the Institute for Medical and Veterinary Science Sequencing Centre, Adelaide, Australia and viewed on the Editview program (Perkin Elmer).

Reverse Transcription

5 μg total RNA and 100 ng 3'-anchored oligo dT_{12} primer (2.2.8) in a total volume of 12 μl was denatured at 70°C for 10 min before snap cooling on ice. The RNA was reverse transcribed using Superscript II RNase H^- reverse transcriptase according to the manufacturer's instructions.

Reverse Transcription PCR (RT-PCR)

cDNA template was diluted 1/10 and 1 ml added to a PCR mix which contained 100μg of each primer and Platinum PCR supermix in a total volume of 219μl in accordance with manufacturer's instructions. The reactions were "hot started" at 94°C for 30 sec to denature the template and activate the enzyme. Typical thermal cycling parameters were: (1) 94°C for 10 sec; (2) 55°C for 10 sec; (3) 72°C for 3 min per kb; (4) cycle 25-35 times. PCR reactions were amplified in a Corbett Research FTS-1 thermal cycler.

Differential Display PCR

2 μl of the reverse transcription mix was used as template in 20 μl PCR reactions containing 1 x Taq buffer, 1.8 mM $MgCl_2$, 250 μM each dNTP, 100 ng

3'-anchored oligo dT_{12} primer, 100 ng arbitrary 10mer, 5 mCi [α-^{33}P] dATP and 1 U *Taq* DNA Polymerase. The ddPCR reactions were amplified in a MJ Research PTC-100 thermal cycler for 40 cycles of: (1) 94°C for 1 min; (2) 42°C for 1 min; (3) 72°C for 30 sec followed by a final extension at 72°C for 5 min. 4 µl samples were removed after amplification, mixed with 4 µl 90% formamide / dye solution, heated to 65°C for 5 min and run on 6% polyacrylamide sequencing gels. After electrophoresis, the gels were transferred to 3MM Whatmann paper, dried and secured down in an autoradiographic cassette. Radioactive ink was dotted onto the Whatmann paper to allow alignment of the film with the gel after exposure. X-ray film was placed in the cassette and the gel was exposed to film for 12 and 24 hours. Radioactive pUC and SPP-1 markers were used as markers of product size.

To reamplify ddPCR products of interest, 26G needle holes were punched in the X-ray film surrounding the individual band. The film was carefully realigned with the gel using the radioactive ink dots and the position of the ddPCR product was marked through the holes with a fine pencil. A gel / Whatmann slice was excised from the marked region of the dried gel and the ddPCR product was eluted in 100-200 µl of elution buffer at 50°C O/N. 5 µl of eluted ddPCR product was then used directly as a template in 100 µl PCR reamplification reactions under the same conditions as the original ddPCR, omitting radioactive ^{32}P label. 25 µl of the reamplified product was purified by agarose gel electrophoresis and Bresaclean. The purified PCR product was then ligated into pGEM-T easy.

Isolation of RNA from Embryoid Bodies

Embryoid bodies were harvested and stored at -20°C until use (a maximum of 3 weeks). RNA was isolated using the RNAzol B method in accordance with the manufacturer's instructions. The RNAzol B method is an improvement of the original method described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Briefly, samples were homogenized using acid-washed glass beads and RNA extracted by the formation of hydrophilic RNA complexes with guanidinium. The hydrophilic interactions of DNA and proteins were effectively abolished,

efficiently removing DNA and proteins from the aqueous phase, whilst RNA remained in this phase allowing its isolation. The RNA was then precipitated and the pellet resuspended in an appropriate volume of water. The concentration of RNA was determined by spectrophotometry and the integrity of RNA was detected by agarose gel electrophoresis. RNA samples were stored at -20°C.

RNA gels for Northern Blot Analysis

1% agarose gels for northern blot analysis were prepared by dissolving 2.5 g agarose in 210 ml MQ water. Once the gel solution had cooled to 60°C, 25 ml 10 x MOPS and 15 ml 20 % formaldehyde (freshly prepared by dissolving 4 g paraformaldehyde in 20 ml MQ water) were added before pouring the gel. 20 μg RNA was prepared for electrophoresis in 50 μl samples containing 1x MOPS, 6.5% formaldehyde (pH 4.5) and 50% deionised formamide. RNA samples were denatured by heating at 65°C for 15 min and snap cooled on ice. GLB was added before loading into wells. 5 μg *Eco*RI digested SPP-1 phage markers were also loaded. Northern gels were run at 6 V/cm gel length in 1 x MOPS until the bromophenol blue dye had reached the bottom of the gel.

Northern Blot Transfer

The lane containing the DNA markers was removed from the gel, stained in EtBr for 45 min and destained in water O/N before photographing under medium wavelength UV light. The remainder of the gel was blotted onto Hybond-XL nylon membrane using capillary transfer. Two pieces of Whatmann 3MM paper were pre-wetted in 20 x SSC and placed over a platform so that the edges of the paper were submerged in 20 x SSC. The gel was orientated with wells facing down on the damp Whatmann paper (avoiding bubbles) and parafilm placed around the gel to avoid short circuiting. The nylon membrane was pre-wetted in 20 x SSC and then carefully placed over the gel such that no air bubbles were trapped between the filter and the gel. Two pieces of Whatmann paper, pre-wetted in 20 x SSC, were then placed on top of the membrane, followed by a 1 cm stack of dry Whatmann paper. A 5 cm stack of paper towels was then added and a glass plate placed on top of the paper towels. A 0.8 kg weight was placed on the glass plate

and the gel was transferred for at least 24 hours. Following transfer, the RNA was cross-linked to the filter by exposure to 120 mJ of UV radiation in a Stratagene UV Stratalinker™ before pre-hybridisation.

RNase protection analysis

RNase protection assays were performed and *mGAP* riboprobes generated as described in Chapman et al. (1997). A riboprobe for the detection of *Sox1* was transcribed from pR1X*Sox1*, a derivative of plasmid #1022. Plasmid #1022 contains 1100 bp of the *Sox1* cDNA in Bluescript KS (obtained from Dr. R. Lovell-Badge, Division of Developmental Genetics, National Institute for Medical Research, Mill Hill, London). A ~360 bp *EcoRI*/*XhoI* *Sox1* fragment from #1022 was subcloned into BluescriptII KS+ (Stratagene) to generate pR1X*Sox1*. *Sox1* antisense probes were generated by digestion of pR1X*Sox1* with *BamHI* and transcription with T3 RNA polymerase.

Radioactive DNA probes

DNA probes were synthesised using the Gigaprime labelling kit with 50 μCi [α-^{32}P] dATP in 24 μl reactions. The reactions were made up to 100 μl with MQ water and unincorporated label was removed from the probe by centrifugation on a Sephadex G-50 column (Amersham Pharmacia Biotech Inc.) centrifuged at 3000 rpm for 5 min.

Radioactive RNA probes

Riboprobes were synthesised as described by Kreig and Melton (1987), using 60 μCi [α-^{32}P] UTP. Unincorporated label was removed using a Sephadex G-50 column centrifuged at 3000 rpm for 5 min.

Hybridisation of Radioactive Probes to Nylon Filter

DNA probes

Filters were prehybridised in 7 ml UltraHyb (Ambion) for a minimum of 4 hours at 42°C in a Xtron hybridisation oven. DNA probes were heated to 100ºC for 5 min and snap-cooled on ice before adding to the filters. Filters were probed O/N at 42°C and the radioactive filter washed the next day in 2 x SSC, 0.1% SDS for 15 min at 42°C followed by 3 x 15 min washes of 0.2 x SSC, 0.1% SDS at 42°C.

RNA probes

Northern filters were prehybridised for a minimum of 4 hours at 65°C in 7 ml Ultrahyb (Ambion) in a Xtron hybridisation oven. RNA probes were denatured by heating at 85°C for 2 min and snap-cooled on ice before addition to the filters. Hybridisation reactions were carried out O/N at 65°C. Radioactive filters were washed the following day using the same protocol described for DNA probed filters.

All filters were sealed in clear plastic and the filter exposed for 24 hours in a phosphorimaging cassette and processed on a Phosphorimager (Molecular Dynamics).

Digoxygenin (DIG) Labelled RNA probes

DIG labelled riboprobes were prepared by transcription reactions containing 1-2 μg linearised plasmid, 1 x transcription buffer, 2 U RNasin, 1 x DIG labelling mix (Boehringer Manneheim) and 20 U appropriate RNA polymerase in a total volume of 20 μl. Transcription reactions were incubated at 37°C for 2 hours and the reactions stopped with 2 μl 0.2 M EDTA. The total volume was increased to 50 μl with Ste buffer and the probe isolated using a Sephadex G-50 column centrifuged at 3000 rpm for 5 min. A 1 μl sample of probe was used to assess

riboprobe yield and quality by agarose gel electrophoresis.

***In situ* Hybridisation of Embryoid Bodies**

Embryoid bodies were washed once in PBS and fixed for 30 min in 4% PFA/PBS solution. Embryoid bodies were then dehydrated in 50% ethanol for 30 min and stored in 70% ethanol at -20°C. The *in situ* protocol consisted of a 2 min wash in 70% ethanol, followed by rehydration in H_2O for 5 min and PBS for 5 min at room temperature. Embryoid bodies were then permeabilised with RIPA buffer for 15 min and washed in 5 x SSC for 10 min. Bodies were then equilibrated for 1 h at 60°C in prehybridisation solution containing 50% deionized formamide, 5 x SSC, 50 μg/ml heparin and 100 μg/ml denatured salmon sperm DNA to block any non-specific hybridisation. DIG labelled riboprobes were denatured at 80°C for 10 min, snap cooled on ice and added to fresh prehybridisation solution. Bodies were hybridised O/N AT 60°C. Prehybridisation and hybridisation steps were carried out in a sealed humidified box containing paper towels soaked in 50% formamide, 2x SSC. Post-hybridisation washes were as follows: 2x SSC for 30 min at RT, 2x SSC for 1 h at 65°C and 0.1x SSC for 1 h at 65°C. Finally bodies were equilibrated for 5 min in buffer 1 at RT. Anti-digoxygenin IgG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) was diluted 1:2000 in buffer 1 + 0.5% blocking reagent (BM) and incubated at RT with bodies for 2 h. Embryoid bodies were then washed twice in buffer 1 at RT for 15 min and once in buffer 2 for 5 min. The bodies were developed in buffer 2 containing 0.45 mg/ml NBT, 0.18 mg/ml BCIP and 5 mM levamisole in the dark until purple staining appeared (2 h - O/N). The staining reaction was terminated by rinsing several times with buffer 3. Destaining, if necessary, was achieved by washing bodies in 100% ethanol. Embryoid bodies were viewed and photographed under phase contrast, Hoffman and bright field optics using a Nikon TE300 inverted microscope with Ektachrome 100 slide film.

Phosphoimager Analysis, Autoradiograph Scanning and Image Manipulation

Gels and filters were exposed to Storage Phoshor Screens (Molecular Dynamics) and processed using a Molecular Dynamics PhosphorImager running

the ImageQuant software package. Quantitation by volume intergration was carried out using ImageQuant. PhosphorImager files were manipulated using the Adobe Photoshop™, Canvas™, and MacDraw Pro™ programs.

Photographic slides of *in situ* hybridisation analysis were scanned. Scanned images were manipulated using the Adobe Photoshop™, Canvas™, and MacDraw Pro™ programs and converted to photographic images.

Sequencing Software and Database Searches

Sequencing reactions were read using a digitiser and MacDNASIS software (Hitachi). Contiguous sequence alignments were performed using MacDNASIS.

Sequence searches and alignments were carried out using the BLAST program of Altschul et al. (1990). cDNA sequences were compared to the non-redundant nucleotide data base and expressed sequence tag database, or translated in six reading frames and compared to the non-redundant nucleotide database translated in all reading frames.

Primers against the mouse *nestin* gene were designed using the Primer3 program.

Tissue Culture Methods

Maintenance of ES cells

ES cells were routinely maintained as described by Smith (1991). Cells were cultured in complete ES cell medium at 37°C in 10% CO_2 on gelatinised plates. ES complete cell medium was replaced every second day. Cells were passaged by washing twice in 10 ml PBS and incubating with 1 ml trypsin for 1 min. Trypsination of cells was terminated by the addition of 1 ml ES cell medium and cells were sedimented at 1200 rpm for 4 min before resuspension in 10 ml complete ES cell medium and re-seeding. ES cells were re-seeded (20-40 fold dilution) every 3-4 days. Cells were harvested for RNA extraction as for passaging

but cells were resuspended in PBS and resedimented at 1200 rpm for 4 min. Cells for RNA extraction were stored as a pellet at -20°C until use.

EXAMPLE 1

Differentiation of pluripotent cells to neurectoderm by culture as EBM in the presence of MEDII

ES cells were aggregated as a single cell suspension at a density of 1 x 10^5 cells/ml in bacterial plates in 50% MEDII conditioned medium in DMEM supplemented with 10% FCS, 1 mM L-glutamine and 0.1 mM β-ME at 37°C in 10% CO_2. Medium was replaced every second day and free-floating EBM were split 1 in 2 every 3-4 days. After 7 days, EBM were transferred to 50% DMEM:50% Hams F12 supplemented with ITSS and 10 ng/ml FGF2 for a further 2 days of culture. This results in the lineage specific differentiation of pluripotent cells to neurectodermal lineages as described in Australian patent application PQ7143.

EBM were harvested by spinning at 1200 rpm for 1 min, resuspending in 10 ml PBS and pelleting the bodies for storage at -20°C until use. The nomenclature used for the ES to EPL and neurectoderm cell conversion was based on the number of days bodies were grown in MEDII. The number of days in culture in MEDII was denoted as a superscript; for example 1 day in culture was represented by EBM^1.

Morphology of Embryoid Bodies During Neuronal Differentiation of ES Cells In Vitro

At 2 days of culture, EBM were perceived as a solid ball of cells with individual cells easily discernible (Figure 1A). By day 4 of culture, cells had proliferated rapidly such that the solid mass of cells had enlarged in size and individual cells were no longer discernible (Figure 1B). No primitive endoderm was visible surrounding the bodies. By day 7, the solid ball of cells had converted into an epithelial sheet of cells. Greater than 90% of the cellular aggregates within the EBM population comprised a convoluted cell layer as shown in Figure 1C. At this

stage, EBMs were transferred to 50% DMEM:50% Hams F12 supplemented with ITSS and 10 ng/ml FGF2 for a further 2 days of culture and at day 9, the cell layer appeared to thicken and organise into a psuedostratified epithelial sheet of columnar cells (Figure 1D).

EXAMPLE 2

Gene Expression Profiles

The temporal expression of marker genes specific ICM, pluripotent cells, neurectoderm and nascent mesoderm during differentiation of pluripotent cells as EBM was determined by Northern analysis or RNase protection (Fig. 2). 20 μg total RNA extracted from ES cells and EBM$^{1-9}$ was analysed by Northern blot for the expression of *Rex1* and *brachyury,* and RNA from EBM$^{4-8}$ for the expression of *Oct4*. The expression of *Sox1* was determined by RNase protection of 10 μg of total RNA isolated from EBM$^{4-9}$. The expression of *mGAP* was used as a loading control in both Northern analysis and RNase protections.

Expression of ICM and pluripotent cell markers

Pluripotent cells of the ICM and ES cells are characterised by expression of the zinc-finger transcription factor *Rex1* (Rathjen et al. 1999; Rogers et al. 1990) and the pluripotent cell marker *Oct4* (Rosner et al. 1990). Northern blot analysis of total RNA from ES cells and EBM$^{1-9}$ revealed expression of *Rex1* in ES cells and EBM^{1}. This expression was down regulated in EBM^{2} and undetectable in EBM$^{3-9}$ (Figure 2A). *Oct4* expression was observed in ES cells and EBM$^{1-4}$ (Figure 2B, data not shown) and down regulated to a low but detectable level in EBM$^{5-9}$. These results suggest that upon EBM formation, pluripotent cells lose ICM-like gene expression, as indicated by the loss of Rex1 expression in EBM^{2}. However, pluripotence is maintained until EBM$^{4-5}$, as shown by the continued expression of *Oct4,* indicative of the differentiation of ES cells to a second pluripotent cell population, primitive ectoderm. The down regulation of *Oct4* on day 5 suggests that the cells have lost pluripotence and differentiated.

Expression of markers specific for ectodermal and mesodermal lineages

Brachyury is expressed in nascent mesoderm formed during gastrulation of the mammalian embryo (Herrmann, 1991). Northern blot analysis of total RNA from EBM^{1-9} did not detect the expression of the nascent mesoderm marker, *brachyury* (Figure 2A).

During mammalian embryogenesis, *Sox1* has been shown to be expressed in neurectoderm at the time of neural plate formation and expression is maintained in all undifferentiated neural cells (Pevney et al., 1998). *Sox1* was used as a marker for the formation of ectodermal lineages in EBM. RNase protection analysis of RNA isolated from EBM^{4-9} with an anti-sense Sox1 probe (Figure 2C) revealed up regulation of *Sox1* expression on day 7-9 of EBM development.

In situ hybridisation analysis of EBM^8 and EBM^9 with deoxygenin labelled anti-sense probes for *Sox1* was performed to assess the homogeneity of expression within the cellular aggregates. *Sox1* expression was detected in all cells of EBM^8 and EBM^9 (see Figure 14A) indicating that the majority of cells have differentiated to the ectodermal derivative, neurectoderm. Similarly, analysis of EBM^8 and EBM^9 with deoxygenin labelled anti-sense probes for *brachyury* confirmed the results of the Northern analysis, since *brachyury* expressing cells were not detected within the cellular aggregates (see Figure 18 A-B).

The absence of *brachyury* expression, and the up regulation of Sox1 expression, suggested that differentiation in EBM resulted in the formation of ectodermal lineages at the expense of mesodermal lineages.

Kinetic analysis of Oct4 down regulation and the onset of Sox1 expression

The levels of expression of *Oct4* and *Sox1* in EBM^{4-8} and EBM^{4-9} respectively, were quantitated, expressed as a ratio with mGAP and graphed (Figure 2D). The down regulation of *Oct4* within EBM was seen to be complete by EBM^6. Although low level expression of *Oct4* could be observed in EBM^{6-8}, it was

maintained at a relatively stable level. In contrast, *Sox1*, the earliest marker of neurectoderm, was not up regulated until day 8, suggesting the presence of an intermediate population of partially differentiated cells characterised by a low levels of *Oct4,* but prior to the upregulation of *Sox1*.

Differentiation of EPL cells to Neurectoderm – Existence of a Definitive Ectoderm intermediate

Oct-4 expression was maintained on all days of EBM development but was significantly down regulated from day 5. The low expression maintained during day 5-9 correlated with expression reported *in vivo*. In the embryo, *Oct4* expression is down regulated during gastrulation at 7 dpc and expression is maintained in ectodermal derivatives until 9 dpc (Schöler et al. 1990; Yeom et al. 1996). This suggests that a definitive ectoderm equivalent (DEE) is formed at day 5/6 in the EBM differentiation system.

The expression profile of *Sox1* indicates conversion of the definitive ectoderm to a neurectoderm equivalent population at day 7. In the embryo, *Sox1* is one of the earliest transcription factors to be expressed in cells committed to the neural fate, with the onset of expression coinciding with neurectoderm induction at 7.5 dpc (Pevny et al. 1998) (Figure 3). In situ hybridisation demonstrated that *Sox1* expression was maintained by all cells in the EBMs until at least day 9.

The lack of detectable *brachyury* expression over the entire 9 day transition indicates the absence of mesoderm formation as *brachyury* expression is observed throughout nascent mesoderm underlying the primitive streak in the embryo.

The expression of early embryonic marker genes therefore indicates that lineage specific pluripotent cell differentiation within EBM proceeds via a transient, homogeneous intermediate population with a gene expression profile equivalent to primitive ectoderm in vivo. Figure 3 relates the temporal order of gene expression changes during EBM differentiation *in vitro,* and events *in vivo*. The temporal gene expression pattern observed correlated with embryonic development. The data

indicate a gap in the window of gene expression from day 4 (primitive ectoderm, *Oct-4*$^{+}$) to day 7 (neurectoderm, *Sox1*$^{+}$). Cells at this stage, between day 5 and day 7 of EBM development, are *Oct-4* low, *Rex1*$^{-}$ and *Sox1*$^{-}$ and may represent definitive ectoderm.

EXAMPLE 3

Identification of differentially expressed genes during directed neurectodermal differentiation of pluripotent cells in response to MEDII

Differential Display Polymerase Chain Reaction (ddPCR)

Differential display PCR (ddPCR) (Liang and Pardee 1992) is a powerful method for identification of differentially expressed genes. Each RNA sample is reverse transcribed with an anchored oligo(dT) primer that anneals at the 5' end of poly(A) tails. In combination with a decamer oligonucleotide of arbitrary sequence, which can hybridize to a subset of cDNAs, cDNA fragments representing the 3' termini of mRNAs defined by both primers are amplified. This procedure therefore allows amplification of an mRNA subpopulation without prior knowledge of sequence information.

Identification of Population Specific Markers

The key strategy in this approach was to screen for specific transcripts with expression restricted to the definitive ectoderm cell population postulated to be present during days 5-7, and to exclude transcripts expressed in ES, EPL and neurectodermal cells. In order to achieve this, gene expression within EBM^{0} to EBM^{9} was compared by ddPCR. The EBM$^{0-9}$ series was generated as described in example 1.

RNA samples were reverse transcribed with an anchored oligo-dT$_{12}$ primer and first strand cDNA was PCR amplified with the oligo-dT$_{12}$ primer in combination with one of the six different arbitrary 5' primers (OPERON primer kits OPA and OPB). Figures 4, 5 and 6 show the ddPCR profile of EBM$^{0-9}$ using the

OPB-07, OPB-09 and OPA-17 5' oligonucleotides respectively. Each duplicated banding profile was generated from independent reverse transcriptions, demonstrating consistency in amplification and detection of differential gene expression during differentiation. Furthermore, samples were re-run on a second denaturing gel and displayed the same expression profiles (data not shown). Amplified products were labelled according to the arbitrary 5' primer used (kit "A" or "B" and oligo number) and the differentially expressed band number on the gel.

The majority of primer combinations displayed approximately twenty major products and thirty-five minor products, as defined by low level amplification or blurred banding. This indicated that the expression patterns of up to 4200 transcripts may have been assayed within this analysis. The expression patterns of bands selected for cloning and further analysis are summarised diagrammatically in Figures 7 and 8.

Isolation and Cloning of Differentially Expressed cDNA Fragments

B078, A175, A091, A193, A1712, A191, A205, A1711 and B098 were excised, eluted and reamplified by PCR using the original oligonucleotides. A1711 was renamed A17113A. All products reamplified as single products of the expected size (Figure 10). All reamplified fragments were purified by agarose gel electrophoresis and Bresaclean and ligated into pGEM-T easy vectors. A single clone for each cDNA fragment was sequenced in both directions. All sequences are presented in Appendix I.

B095, B072 and A175 represented cDNA fragments derived from the murine *CD24a* gene (99% homology and 99% identity over 461 nucleotides) (Wenger et al. 1993; Accession no. MMCD24A). All three fragments spanned the same 6939 bp to 7399 bp region in the 3' untranslated region (UTR) of the *CD24a* gene, and resulted from amplification with the oligo dT primer on both strands. The B095, B072 and A175 fragments displayed extremely similar expression profiles in the ddPCR screen (Figure 4; Figure 5; Figure 6).

A178 corresponded to the *p12*$^{DOC-1}$ (Shintani et al. 2000; Accession no.

AF011644) mouse oral tumour suppressor homologue (98% identical over 277 nucleotides) and spanned positions 702 bp to 978 bp of the 3'UTR.

The sequence of the B098 fragment was homologous to the mouse *diazepam-binding inhibitor* (*DBI*) gene (98% identical over 159 nucleotides) (Kato; in press; Accession no. MMDIAZBI) and corresponded to positions 229 bp to 387 bp of the 3'UTR.

B078 corresponded to an uncharacterised murine ribosomal protein L23 (Rp123) mRNA sequence deposited in the database (Kleiter et al. 2000; Accession no. AF287271) with 98% identity spanning 158 nucleotides. The fragment corresponded to positions 319 bp to 475 bp of the mRNA which spanned 121 bp of the 3' end of the open reading frame (ORF).

A1712, A17113A, A091, A191, A193 and A205 were novel sequences, not homologous to any described sequences or functional domains. An Expressed Sequenced Tag (EST) search detected each of these sequences in multiple and diverse mouse cDNA libraries. A17113A expression was detected in mouse ES cell, one-cell and two-cell embryos, and 9-, 12- and 15-day embryonic libraries.

Validation of Expression Patterns by In Situ Hybridisation Analysis of Expression in EBM6 and EBM9

Cloning of transcripts following ddPCR can be complicated by stochastic background amplification products in the primary ddPCR which carry through to reamplification and cloning. This can lead to difficulty when attempting to identify clones which recapitulate the expression profile determined by ddPCR (Callard et al. 1994). It is therefore necessary to validate the expression patterns of individual cDNA clones, thus demonstrating that the desired fragment has been cloned.

In situ hybridisation to EBMs was used to analyse gene expression through EBM development. This technique has the added advantage in that the uniformity of gene expression within bodies can be assessed. Direct visualisation of A175, A1712, A17113A and B072 expressing cells was therefore carried out by *in situ*

hybridisation analysis of EBM^6 and EBM^9. Expression was detected using digoxygenin labelled antisense riboprobes generated from the cDNA clones.

The A175 and B072 transcripts exhibited expression patterns consistent with those observed by ddPCR. Expression was relatively low but detected in all cells in EBM^6 and EBM^9 (Figures 11 A, B, C and D). Expression of the A17113A transcript was detectable in all cells of EBM^6 and was down regulated in EBM^9 (Figure 11 E and F), consistent with the ddPCR expression pattern (Figure 8). Expression of the A1712 transcript was prominent and also in accordance with the expression profile predicted from ddPCR. Strong expression in all cells of the bodies at EBM^6 was followed by down regulated expression in EBM^9 (Figure 11 G and H). The detection of transcript expression in all cells of EBM^6 was consistent with the described homogeneity of the differentiation system.

Discussion

DdPCR was used to identify genes that were expressed in EBM cells equivalent to definitive ectoderm (DEE). The timing of upregulation and downregulation of some of these suggest that they could be used as markers to define the temporal and spatial existence of definitive ectoderm in vitro and in vivo. Verification of expression patterns by in situ hybridisation confirmed the expression patterns demonstrated by ddPCR. Further, expression of the markers in all cells of EBM^6 confirmed the homogeneity of definitive ectoderm formed by directed differentiation of pluripotent cell aggregates in response to MEDII.

EXAMPLE 4

Lineage specific formation of surface ectoderm from ES cell-derived definitive ectoderm in response to Bone Morphogenetic Protein 4 (BMP4)

The developmental potential of definitive ectoderm during mouse embryogenesis has been studied by transplantation and fate mapping experiments (Beddington 1982; Gimlich and Cooke 1983; Hogan et al. 1994). These experiments demonstrate that definitive ectoderm gives rise to two

subsequent embryonic tissue types which are more restricted in their developmental potential: the surface ectoderm and the neurectoderm. Whilst the surface ectoderm predominantly gives rise to such tissues as the epidermis, lens and cornea which contribute to the skin and eye of the embryo respectively, cells of the neurectoderm develop into neurons and glia of the central nervous system (CNS) and sensory neurons, postganlglionic autonomic neurons and Schwann cells of the peripheral nervous system (PNS). Developmental bipotentiality to form these two ectodermal derivatives is distinctive of definitive ectoderm. Demonstration that EBM-derived definitive ectoderm (DEE) is capable of differentiation into both neurectoderm and surface ectoderm, but not mesoderm, would support the proposition that it is representative of definitive ectoderm *in vivo*.

Members of the Transforming Growth Factor-b (TGF-b) gene family are good candidates for molecules involved in surface ectoderm induction. For example, Bone Morphogenetic Protein 4 (BMP4), which is expressed in ventral ectoderm of the midgastrula stage *Xenopus* embryo (Hemmati-Brivanlou and Thomsen 1995), has been shown to be an endogenous neural inhibitor and epidermal inducer in dissociated ectodermal explants (Hawley et al. 1995; Sasai et al. 1995; Suzuki et al. 1995; Wilson and Hemmati-Brivanlou 1995; Xu et al. 1995) and has been implicated in lens induction in the mouse by knockout studies (Furuta and Hogan 1998).

EBM^6 were generated as described in example 1. Early on day 6, bodies were transferred to gelatinised wells and left to adhere in 50% MEDII for 8-10 hours, after which the media was removed and replaced with 50%F12: 50%DMEM supplemented with 1/1000 ITSS and 1/1000 glutamine, conditions which have previously been demonstrated to support homogeneous neurectoderm formation (J. Rathjen; unpublished data). To test the effect of BMP4, 10 ng/ml BMP4 was added to EBM^6 and bodies were cultured until day 12, fixed in 4% PFA and stored at -4°C.

Morphological Alterations in Differentiated Cells

The morphology of the bodies and differentiated cells was observed and documented daily. In the absence of BMP4, cells on the periphery of seeded EBM differentiated and formation of neurectodermal/neural lineages was assessed morphologically on day 12 by the presence of neurons. Bundles of axons were visible in 87.1% of EBM cultured in the absence of BMP4 as depicted in Figure 12A and 12C. The addition of BMP4 to the culture media significantly reduced ($p<0.01$) neuronal differentiation such that only 18.1% of the 44 bodies examined exhibited any morphological sign of neuron formation (Figure 12B; Figure 12C), indicating suppression of neuron formation by BMP4.

EBM^6 cultured for a further 2 days in the absence of BMP4 exhibited morphology typical of neurectoderm differentiated from ES cells in vitro in response to MEDII (example 1). The central mass of cells comprised a convoluted epithelial sheet of columnar cells and individual differentiated cells did not generally migrate far from the outer edge of bodies (Figure 13A and 13B). In contrast, EBM^6 cultured for a further 2 days in the presence of BMP4 displayed a distinct morphology. By day 8, the central mass of cells comprised a convoluted epithelial layer which in general was more dispersed and spread out (Figure 13C). Individual differentiated cells derived from the bodies migrated a larger distance from the centre of the bodies and adopted an alternative morphology, arranged as a simple epithelial layer of cuboidal or squamous cells (Figure 13D). Cells contained large cytoplasms and were organised in a cobble-stone manner. Cell-cell boundaries were relatively tight and the surface of cells appeared textured and ruffled. This morphology, which was never observed in EBM cultured without BMP4, is reminiscent of the appearance of early epidermis (Karp and Berrill 1981).

Gene Expression in Differentiated Cells

The loss of terminal neuron differentiation and the formation of a distinct cell type reminiscent of early epidermis or surface ectoderm, in response to BMP4, was investigated further by analysis of epidermal and neurectodermal

marker expression. EBM^6 cultured as described above were fixed in 4% PFA on days 7, 8 and 9, dehydrated and stored in 70% ethanol at -20°C prior to gene expression analysis by *in situ* hybridisation for the expression of *Sox1*, *nestin*, *K18*, *K14* and *brachyury*.

Sox1, a marker which delineates the neural plate and is expressed by all undifferentiated neural cells and *nestin*, which encodes an intermediate filament protein expressed in the developing central nervous system (CNS) from 7.75 dpc in the neural plate and maintained in many proliferating CNS progenitor cells (Dahlstrand et al. 1995), were used to assess the formation of neurectoderm in EBM. *Sox1* (Figure 14A) and *nestin* (Figure 15A) expression was detected in all cells of EBM^6 cultured for a further 2 or 3 days in the absence of BMP4. In the presence of BMP4, *Sox1* expression was significantly reduced with little expression being detected in any of the cells surrounding the bodies (Figure 14B). Expression of *nestin* was also reduced by ectopic BMP4 however a low but constant level was maintained (Figure 15B and C). This suggests that addition of BMP4 directs differentiation of definitive ectoderm away from a neural/neurectodermal fate.

Formation of surface ectoderm was assessed by the expression of keratin intermediate filaments, the composition of which is specific for particular types of epithelial differentiation and development (Lane and Alexander, 1990). *Keratin 18* (*K18*) is expressed early in embryonic development (Oshima et al. 1986) and serves as a marker for simple epithelium. *K14* expression is up regulated later during development by mitotically active keratinocytes of the epidermis which can undergo stratification (Fuchs and Green 1980; Sinha et al. 2000). *K18* and *K14* expression were not detected by *in situ* hybridisation in the absence of BMP4 (Figure 16A; Figure 17A) with background staining equivalent to *in situ* hybridisation using the sense probes (data not shown). This indicates that surface ectoderm is not formed during standard EBM differentiation, consistent with the described homogeneity of neurectoderm production. In contrast, expression of both *K18* and *K14* was up regulated when EBM^6 were cultured with BMP4 and was detected in the squamous differentiated cells with an early epidermal

morphology (Figure 16 B and C; Figure 17B).

Formation of mesoderm can be detected by expression of *brachyury*, a transcription factor up regulated in nascent mesoderm (Herrmann 1991). *Brachyury* transcripts were not upregulated in either culture condition (Figure 18A and B), confirming the ectodermal lineage specificity of EBM differentiation.

Discussion

The expression of epidermal markers and reduction in neural marker expression, in conjunction with the absence of *brachyury* expression and prominent epithelial morphology suggests that definitive ectoderm derived by differentiation of pluripotent cells in the presence of MEDII differentiates in response to BMP4 to form a surface ectoderm equivalent population. This bipotential differentiation provides direct evidence that the starting population was representative of embryonic definitive ectoderm.

EXAMPLE 5

Lineage specific formation of surface ectoderm from ES cell-derived definitive ectoderm in response to Epidermal Growth Factor (EGF).

Epidermal Growth Factor (EGF) has been proposed to play an important role in epidermal development in the adult mouse by promoting proliferation and differentiation (keratinization) of the epidermis, and is required for the culture of epithelial cells *in vitro* (Partanen 1990; Sibilia and Wagner 1995).

EBM^6 were generated as described in example 1. Early on day 6, bodies were transferred to gelatinised wells and left to adhere in 50% MEDII for 8-10 hours, after which the media was removed and replaced with 50%F12: 50%DMEM supplemented with 1/1000 ITSS and 1/1000 glutamine, conditions which have previously been demonstrated to support homogeneous neurectoderm formation (J. Rathjen; unpublished data). The effect of EGF on cell differentiation was tested by addition of 10 ng/ml EGF, 10 ng/ml EGF + 10 ng/ml BMP4, or

10ng/ml BMP4 to EBM[6] which were cultured until day 12, fixed in 4% PFA and stored at -4°C.

Morphological Alterations in Differentiated Cells

The morphology of individual bodies cultured in the absence of EGF was as described previously. In the presence of EGF, individual differentiated cells surrounding the central aggregate displayed a morphology similar to that observed following BMP4 addition. Cells were arranged as a simple epithelial layer of cuboidal or squamous cells. Compared to addition of BMP4 alone, cell-cell boundaries in EGF-induced cells were much tighter and the textured, or ruffled appearance of cells was no longer visible. The cobble-stone arrangement of cells was more apparent and cells in general appeared more epidermal-like (Figure 19F; Figure 20C; Figure 22E) (Karp and Berrill 1981). EBM cultured in the presence of BMP4 + EGF formed cells with morphology characteristic of cells induced in response to BMP4 or EGF alone (Figure 19G-H; Figure 21H; Figure 22G). Neurons were not observed following differentiation of EBM in the presence of EGF or BMP4/EGF.

Gene Expression in Differentiated Cells

The expression of epidermal, mesodermal and neurectodermal markers was investigated as described in example 4. In the presence of EGF, expression of the neural marker, *Sox1*, was significantly reduced with little or no transcript expression detected (Figure 19A-B; Figure 19E-F). The presence of EGF + BMP4 in the culture media also served to reduce *Sox1* expression to levels analogous to those seen with BMP4 alone (Figure 19G-H). *Nestin* expression was completely abolished by addition of EGF (Figure 20C) and strongly reduced by the combination of EGF + BMP4 (Figure 20D) to levels observed by BMP4 addition alone (Figure 20B). The reduction of *Sox1* and *nestin* expression indicated divergence from the neurectoderm lineage.

Although addition of BMP4 and BMP4/EGF increased expression of the early epithelial marker *K18* in differentiated cells, differentiation in the presence of

EGF did not significantly augment *K18* expression (Figure 21A-H). In contrast, the later stage epidermal marker, *K14*, exhibited dramatic up regulation (Figure 22C-E). The extent of *K14* expression appeared higher than that detected in the presence of BMP4 (Figure 17B) or BMP4/EGF (Figure 22F-G). *Brachyury* transcripts were not detected within any of the culture conditions (Figure 23A-D), confirming that no mesoderm formation had occurred.

Discussion

Up regulation of the epidermal marker *K14* and down regulation of the neural markers *Sox1* and *nestin* suggest that EGF induces differentiation of definitive ectoderm, formed by differentiation of pluripotent cells in response to MEDII, to surface ectoderm. The fact that *K18* expression was not detected may indicate that differentiation into epidermis is more advanced in the presence of EGF than BMP4, or that a distinct surface ectoderm differentiation endpoint is induced by EGF. In conjunction with the absence of *brachyury* expression, these data confirm the bipotential ectodermal differentiation of the starting population, consistent with designation of EBM[6] as equivalent to definitive ectoderm in vivo.

APPENDIX 1

A091

ATGAATTCTT	TTTTTTTTTT	AATTTATAAA	GTTTTATTTT	TGTATATGTG	CTAGATCTTA	60
CGAACTGCGG	ACAATCTTTA	TGTCAATCTG	TTGGCTTGTA	AAAGGCTAAG	AAGTATGTGA	120
GATTTGGGCN	GGTGAAATTT	CCTTCTGGGA	ACCCCAATTC	TAAAAAAAAA	AAAGAATTCA	180
T						181

A17112A, A175, B072, B095

ATGAATTCTT	TTTTTTTTTT	AAGAGAAGAA	CAAACGGTGG	ATCAGAATAG	CCACGTTTGG	60
AATACTTTGG	TTATCCATTC	ATATTTTTAG	ATAGTTATTG	GTCCTGTGCC	TGAAAGGGGG	120
CTTGGTTCTA	CCGTAAGTTT	TTCCAATTTC	CTTGATATAC	ACATACCTTC	TAAAACCTAG	180
ACATTTCCTG	AAAAAAATCT	TTTGTTCGCA	TGGTCACACA	CTGATGCTTA	CCCGTACAGT	240
AGTCTTGATA	ACCAGAGTCA	TTTTCTCCAT	CTTTAGAAAC	CTTCCTGGGA	AGAAGGAGAG	300
CTCACAGACC	CGAAGCTACT	GTGTGTGTGA	ATGAACACTC	CCCTTGCCTC	ACACCTGAAT	360
GCTGTACATC	TATTTGATTG	TAAATTGTGT	TTGTGTATTT	ATGCTTTGAT	TCATAGTAAC	420
TTCTCATGTT	ATGGAATTGA	TTTGCATTGA	ACACAAACTG	TAAAAAAAAA	AAGAATTCAT	480

A17113A

ATGAATTCTT	TTTTTTTTTT	AAAGAAAATA	AGGGATGTGA	ATTTGTATAA	AAAATAACAT	60
CTATCTGTGT	ATACGTAGAA	AGAAGCTGCT	TTTTAAAGAA	CTTCTAATTG	GAAACTCATG	120
TTTTAACCAA	ATTTTAAGGA	TTAATGGAGC	AATCATGGGA	GGGAAGAACA	CAATACAACT	180
ATGCAGGTTT	TATAAATGTG	CAATAAAAGT	ATTTGTTTTA	CCTTTGGTAT	GTAAAAAAAA	240
AAAAGAATTC	AT					252

A1712

GACCGCTTGT	CCAACCATGA	TTACAAAGCA	AACTTTCTGT	CTTAAAAACA	AAACGAAAGG	60
GCTGGAGAGA	TGCTCAGCGG	TAAGAGCACT	GACTGCTCTT	CCAGAAATCC	TACGTTCAAT	120
TCTCAGCAAC	CACATGGTGG	CTCACAACCA	TCTGTAATGG	CGATCTAATG	CCCTCTTTTG	180
GTGTGTCTGA	AGACAGCTAC	AGTGTACTCA	TATACATAAA	ATAAATCTTT	AAAAAAAAAA	240
AAGAATTCAT						250

A178

GACCGCTTGT	GTACAGTGTA	GACCCTGCTT	CATGGAGCTT	CTCTGCCTAA	GCGTTTGCAT	60
GACTGAGTGC	TTGGAAGTCA	ATCTTTAAAA	TGCACAGGTT	ACACATGCAG	AAGAAAGAGC	120
CATCTACCCA	ACCTCCCAAG	CACCCTGCAG	ATGTCCATCC	TGAGGCTGTA	GATCTCAGTT	180
CTGTGTTTAC	TGTGAGCCCA	TCACAACATC	TGGAAGAAAG	CGACTGAAAC	TGTTGCATCT	240
TTGTATTTAT	TACTTGATGT	AATAAATCTT	ATTTTCATTA	AAAAAAAAAA	AGAATTCAT	299

A191

CAAACGTCGG	GGTATCTTTA	TCTTATGTTT	TCTAATGGAA	GATGGCAGCC	CAGAATCCTC	60
AAGACAAAGC	AAGGGGTATC	AGCTGATGGC	CGCGGGACTG	ATGGGTGTCC	TGGGTTCTTT	120
AGCTCACCAG	CACTTTGCGC	TCAATGGACT	TTGTAAATTC	TCATCAGCTG	TTGTAGAAGT	180
TCCTCTGCCT	GGAGCTGTCA	TCTCAGCCGC	TCCCTTGGAA	AGTGCCCCTC	AACGGACCCT	240
GTTCACTGCT	GCACTTCTCA	ATGAATTAAA	ACTGACTTCT	GCTCTAAAAA	AAAAAAGAAT	300
TCAT						304

A193

CAAACGTCGG	GACCCGTTGC	TGTTGAACAG	TGGAGGAAGT	GTTAAGGTAC	TGTAGTGGTT	60
GAGATCGTCT	CTGGTCAACT	TACAGGGAAT	CTTCGCTTTT	AATTTTTGAA	AATTGAATAT	120
TTTATATTAA	ATAAAAGATG	TTTGGTGTGT	AAAAAAAAAA	AAGAATTCAT		170

A205

GTTGCGATCC	CCAGGCGATG	GAGTTGGAGT	CTGAGGAGTA	GTGGGAAATC	ATCCAGGCCC	60
AAGCTACGGG	ACCCAAGATC	AATTCAAACA	TCAGGACTCC	CTAGTGCTGG	CTTCAGAGCG	120
GATGTTTTCT	ATCTCTGTTG	TCTCCATCCC	ATAGCGTAGG	CATTCACAGC	CCACTTGAGT	180
TCCTATCTAA	CTGCCCAGTA	CACTCGCAGC	CGGGTTTTTC	TCTAGGCCAG	AACCTGGTCT	240
TGTGTTCTCT	CTGTTCTGTT	ATTAAAGAGC	ACCAAATAGT	AAAAAAAAAA	AAGAATTCAT	300

B078

GGTGACGCAG	GGGTCATAGT	AAACAATAAA	GGAGAGATGA	AAGGCTCTGC	TATCACNTGG	60
TCCAGTGGCA	AAGGAGTGTG	CAGACTTGTG	GCCCAGAATT	GCATCCAACG	CAGGCAGCAT	120
TGCATGATTC	TCCAGTGTAT	TTGTAAAATA	TATTCATTAA	AGNCTCTGCT	CTAAAAAAAA	180
AAAAGAATTC	AT					192

B098

TGGGGGACTC	GTGGAACAAG	CTGAAAGGGA	CTTCCAAGGA	AAGTGCCATG	AAGACCTATG	60
TGGAAAAGGT	AGACGAGCTA	AAGAAGAAAT	ACGGAATATA	AATCACCAGA	TTTGGTGGCC	120
AGCCACACGT	GTGACCTGTG	AGGACATAAT	GCCTTGGTTT	TTTCTAATGT	AGATGATATG	180
GCTGTGATAC	ATTAGGGCCA	GCGTTAACCT	CTGCTCCTCC	TCCCTCTGTA	GTTTTTACCT	240
ACAATCAATT	AAAAGTACAT	TTGTTACTCT	GTGCCTGGGT	TAAAAAAAAA	AAAGAATTCA	300
T						301

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It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

It will also be understood that the term “comprises” (or its grammatical variants) as used in this specification is equivalent to the term “includes” and should not be taken as excluding the presence of other elements or features.

CLAIMS

1. A method for preparing definitive ectoderm equivalent (DEE) cells *in vitro*, which method includes

providing

a source of early primitive ectoderm-like (EPL) cells; and

a conditioned medium as hereinbefore defined; or an extract therefrom exhibiting neural inducing properties; and

culturing the EPL cells in the conditioned medium or extract for a time sufficient to permit controlled differentiation to definitive ectoderm equivalent cells.

2. A method according to Claim 1, further including the preliminary steps of

providing

a source of pluripotent cells;

a source of a biologically active factor including

a low molecular weight component selected from the group consisting of proline and peptides including proline and functionally active fragments and analogues thereof; and

a large molecular weight component selected from the group consisting of extracellular matrix portions and functionally active fragments or analogues thereof, or the low or large molecular weight component thereof;

contacting the pluripotent cells with the source of the biologically active factor, or the large or low molecular weight component thereof, to produce early primitive ectoderm-like (EPL) cells.

3. A method according to Claim 2, wherein the pluripotent cells are selected from one or more of the group consisting of embryonic stem (ES) cells, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells, EG cells, teratocarcinoma cells, EC cells, and pluripotent cells derived by dedifferentiation or by nuclear transfer.

4. A method according to Claim 1, wherein the conditioned medium is

MEDII.

5. A method according to Claim 1, wherein the neural inducing extract, excludes the biologically active factor, or the large or low molecular weight component thereof.

6. A method according to Claim 1, including the further step of identifying the DEE cells by procedures including gene expression profiles and differentiation potential.

7. A method according to Claim 6, wherein the conversion of EPL cells to DEE cells may be characterised by

down regulation of *Oct4* expression relative to embryonic stem (ES) cells;
substantial absence of neurectoderm marker expression; and
expression of DEE marker genes are hereinafter described.

8. A definitive ectoderm equivalent (DEE) cell derived *in vitro*.

9. A DEE cell according to Claim 8, characterised in being capable of controlled differentiation into neurectoderm or surface ectoderm cells but not mesoderm cells.

10. A DEE cell according to Claim 8, exhibiting two or more of
down regulation of *Oct4* expression relative to embryonic stem (ES) cells;
substantial absence of neurectoderm marker expression; and
expression of DEE marker gene.

11. A marker gene for embryonic definitive ectoderm or a definitive ectoderm equivalent (DEE) cell, the marker gene exhibiting expression in DEE cells.

12. A marker gene according to Claim 11, wherein the marker gene exhibits up regulation of expression on day 4, 5 or 6 after initiation of culturing of ES cells in conditioned medium, as hereinbefore described, and/or down

regulation of expression on day 7 or 8 after initiation of culturing.

13. A marker gene according to Claim 12, selected from one or more of the group consisting of CD24Q, DBI, P12$^{DOC\text{-}1}$, Rp123, A1712, A17113A, A091, A191, A193 and A205 and functionally active fragments or analogues thereof, and molecules which compete therewith for biological activity.

14. A gene sequence selected from one or more of the group consisting of A1712, A17113A, A091, A191, A193 and A205 and functionally active fragments or analogues thereof and molecules which compete therewith for biological activity.

15. A method for selectively producing neurectoderm cells or surface ectoderm cells from DEE cells, which method includes

providing

a source of DEE cells as hereinbefore described, and

a suitable culture medium;

a growth factor capable of promoting development of surface ectoderm; and

further culturing the DEE cells in the presence or absence of the growth factor, and optionally in the presence of additional growth factors and/or differentiation agents, to produce, in the presence of the growth factor, surface ectoderm cells, and in the absence of the growth factor, neurectoderm cells.

16. A method according to Claim 15, wherein the growth factor is selected from a one or more of Transferring Growth Factor-β (TGF-β) or an Epidermal Growth Factor (EGF).

17. A method according to Claim 16, wherein the growth factor is Bone Morphogenetic Protein 4 (BMP4), Bone Morphogenetic Protein 2 (BMP2) or Activin.

18. A method according to Claim 17, wherein the or each growth factor is present in amounts of approximately 5 to 50 ng/ml.

19. A method according to Claim 16, wherein the suitable culture medium includes DMEM and Ham's F12 nutrient mixture.

20. A method according to Claim 19, wherein the suitable culture medium further includes insulin, transferrin and/or sodium selenite.

21. A surface ectoderm cell derived *in vitro* and characterised by two or more of

early epidermal-like morphology;

expression of Keratin 18 (K18); and/or Keratin 14 (K14); and

substantial absence of expression of *Sox1* and nestin.

22. A method for producing genetically modified definitive ectoderm equivalent (DEE) cells which method includes

providing

a source of early primitive ectoderm-like (EPL) cells; and

a conditioned medium as hereinbefore defined; or an extract therefrom exhibiting neural inducing properties; and

culturing the EPL cells in the conditioned medium or extract for a time sufficient to permit controlled differentiation to DEE cells; and

modifying one or more genes in the DEE cells; or

modifying one or more genes in the EPL cells prior to culturing in conditioned medium or extract, to produce generitally modified definitive ectoderm equivalent (DEE) cells.

23. Use of genetically modified or unmodified definitive ectoderm equivalent (DEE) cells according to any one of Claims 8 to 10 or their differentiated or partially differentiated progeny for use in human cell therapy or transgenic animal production.

24. Use of genetically modified or unmodified definitive ectoderm equivalent (DEE) cells according to any one of Claims 8 to 10 or their differentiated or partially differentiated progeny for use in human or animal gene therapy.

25. A method for the preparation of tissues or organs for transplant, which method includes

providing surface ectoderm cells according to Claim 18;

culturing the surface ectoderm to produce skin cells, lens or corneal cells of the eye, or hair cells; and

transplanting the cells produced to a selected site within the patient's body.

26. A method according to Claim 1 or 15, substantially as hereinbefore described with reference to any one of the examples.

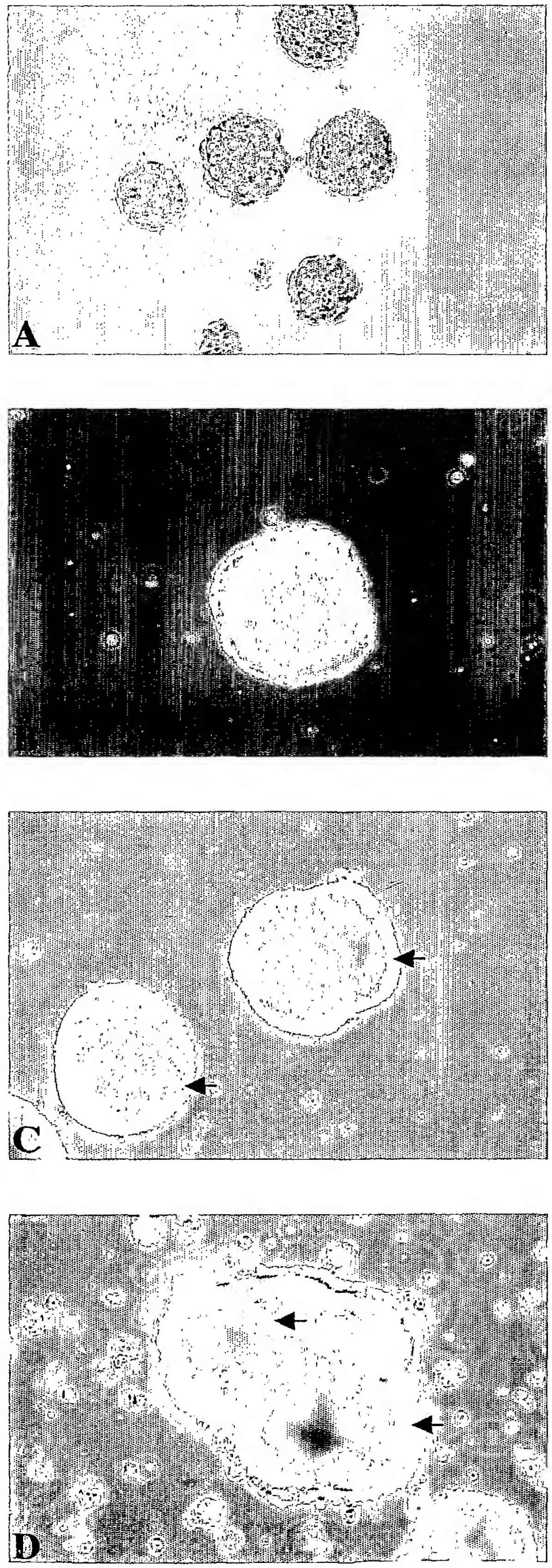
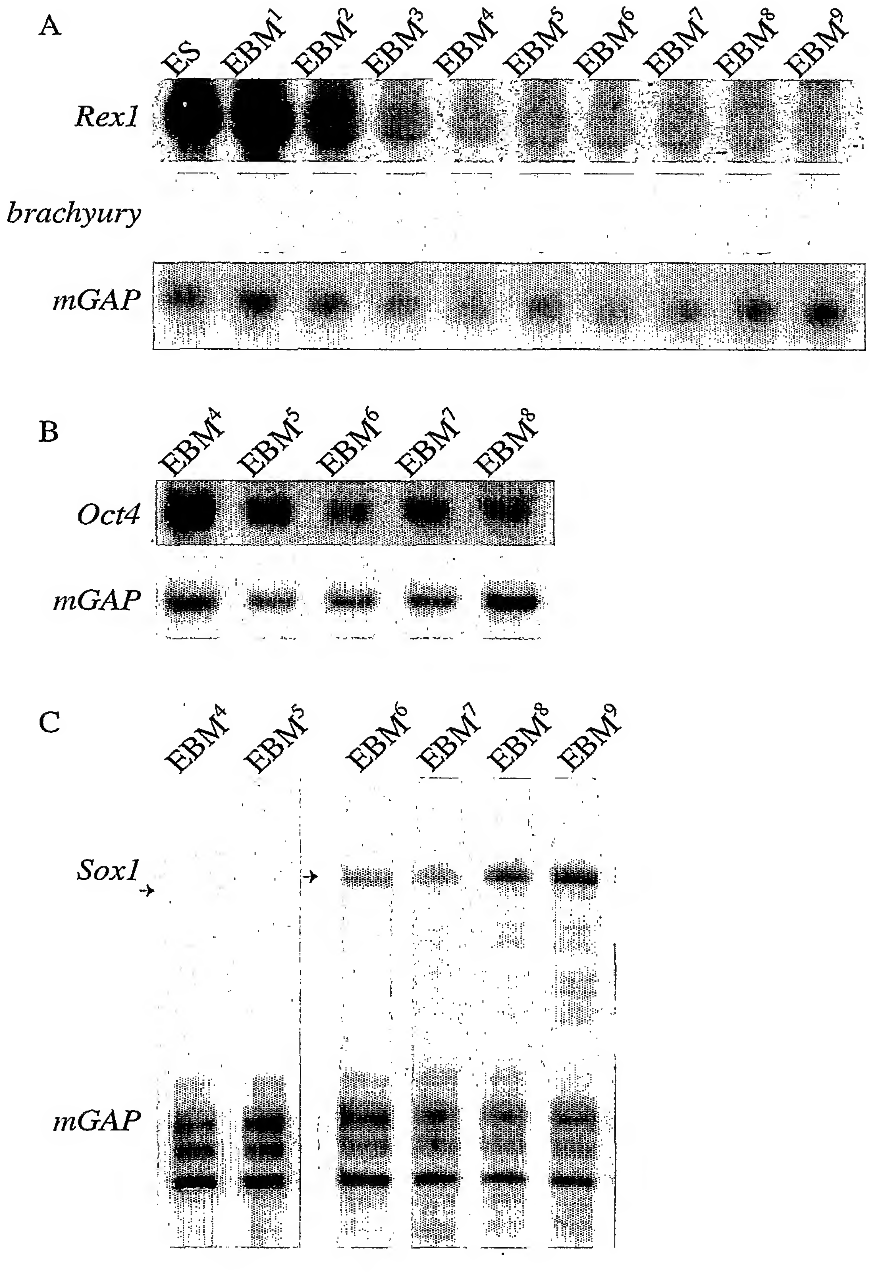
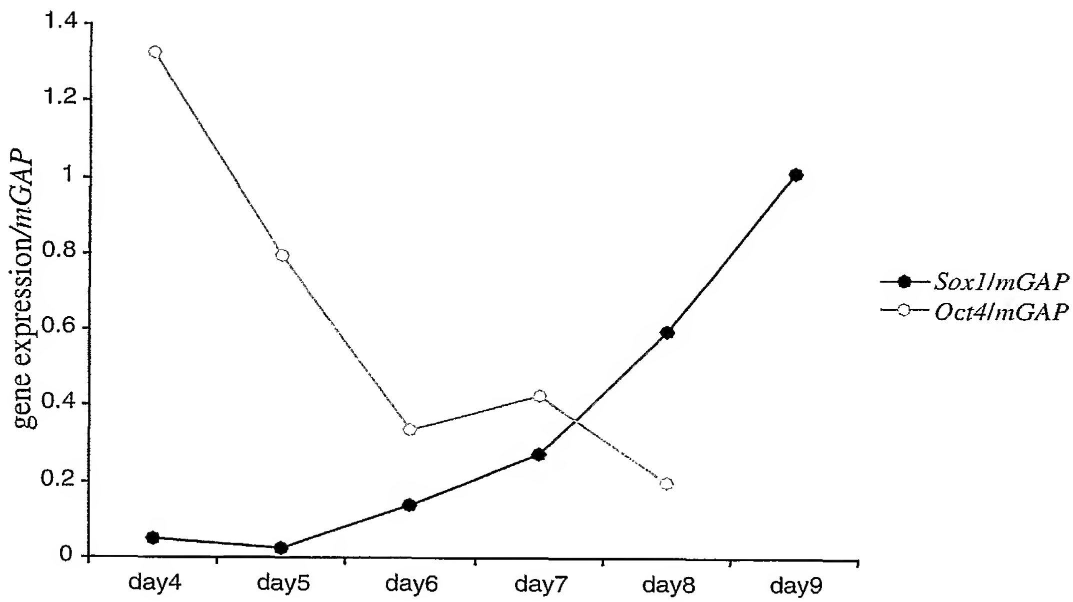


FIGURE 1



FIGURES 2A, 2B, 2C

D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1
0.8
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day9
Sox1/mGAP
Oct4/mGAP

FIGURE 2D

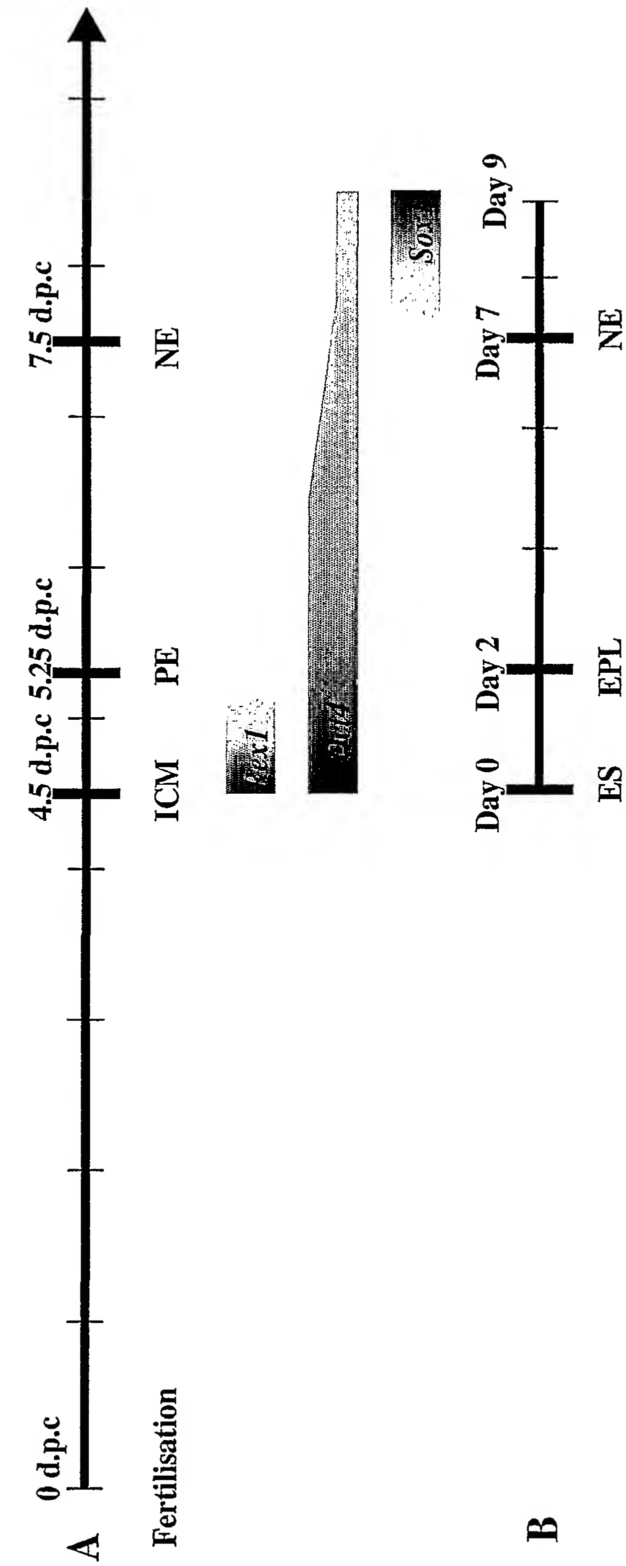
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FIGURE 3

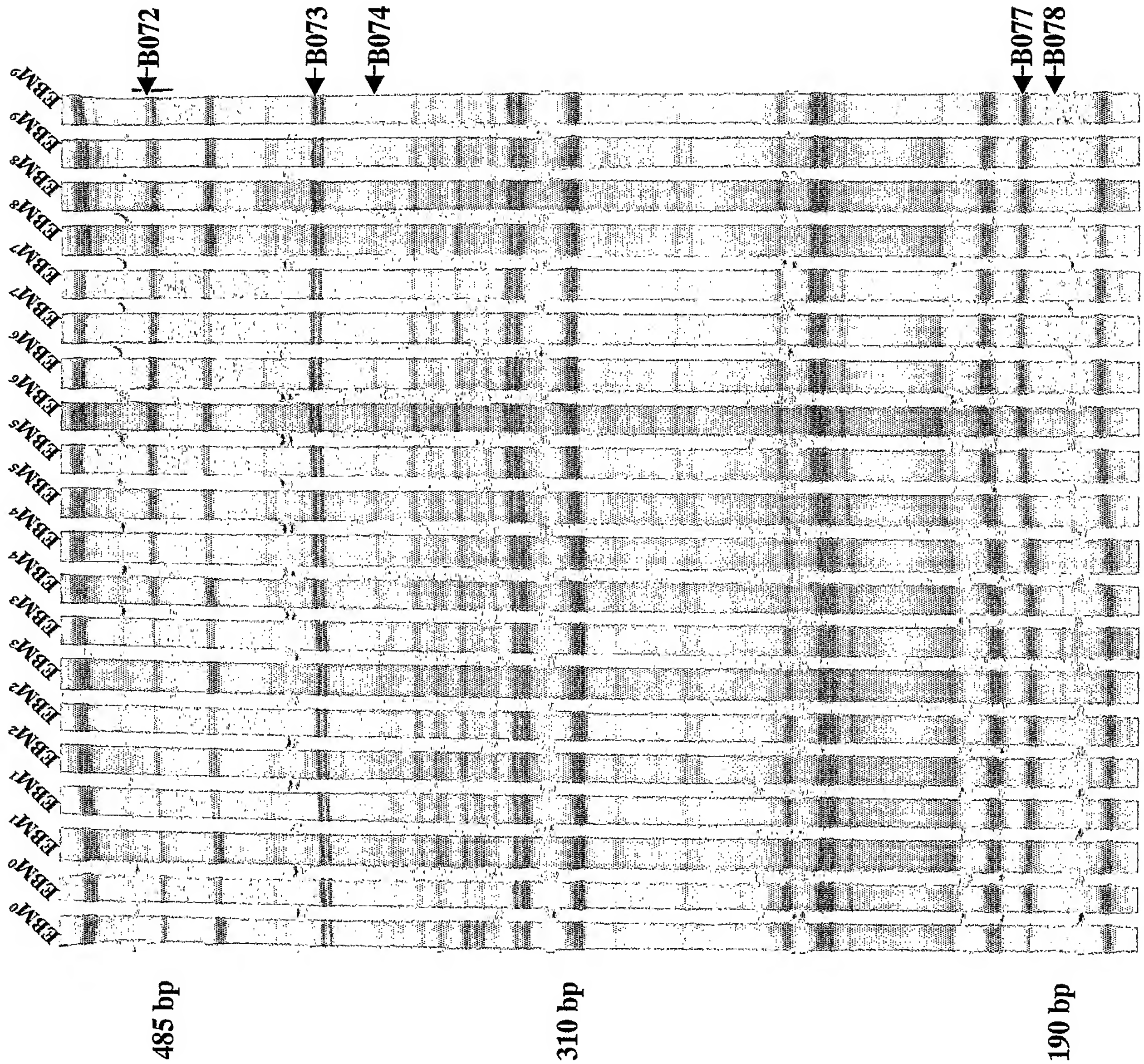
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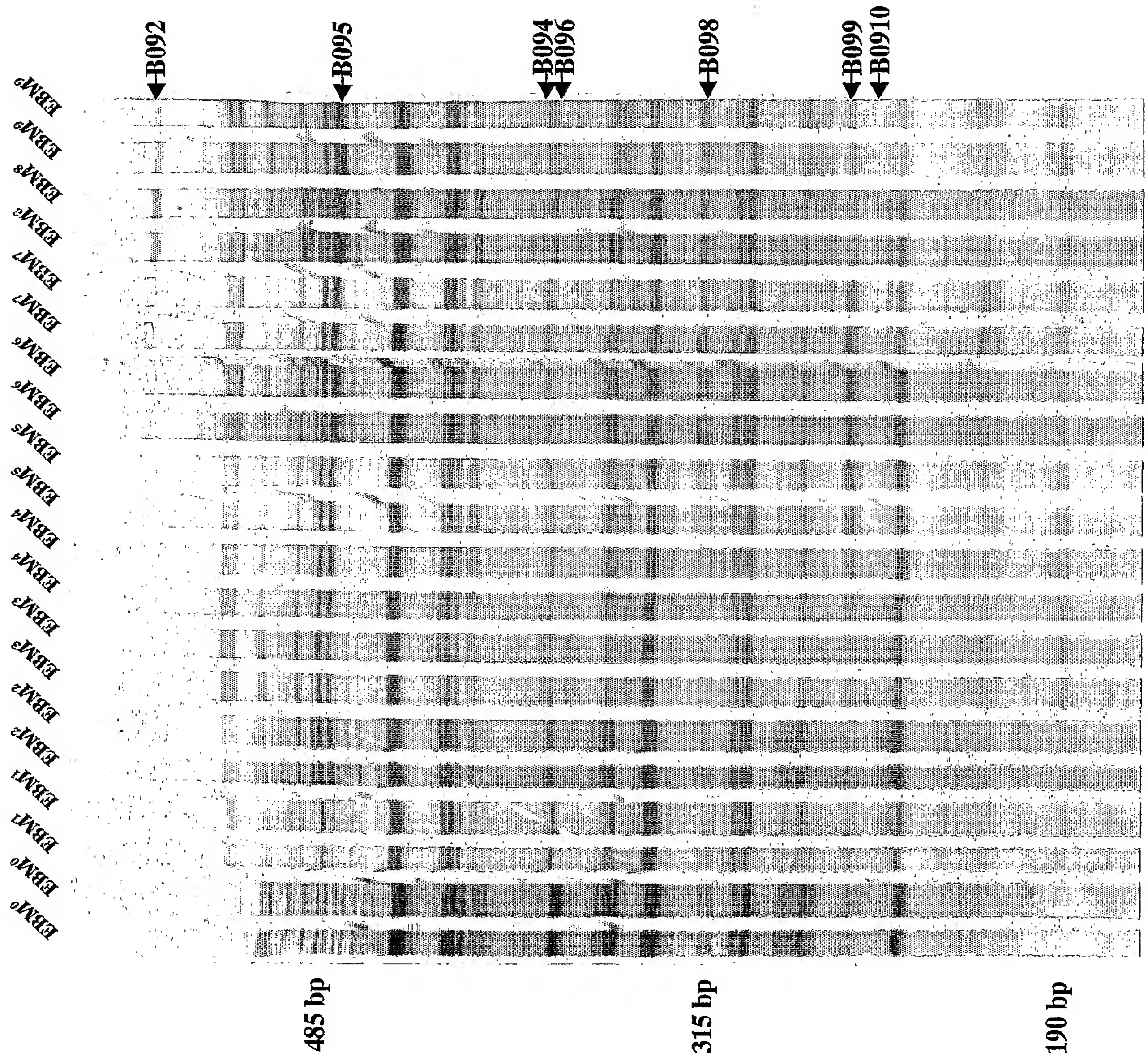
FIGURE 4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EBM7
EBM7
EBM6
EBM6
EBM5
EBM5
EBM4
EBM4
EBM3
EBM3
EBM2
EBM2
EBM1
EBM1
EBM0
EBM0
485 bp
315 bp
190 bp

FIGURE 6

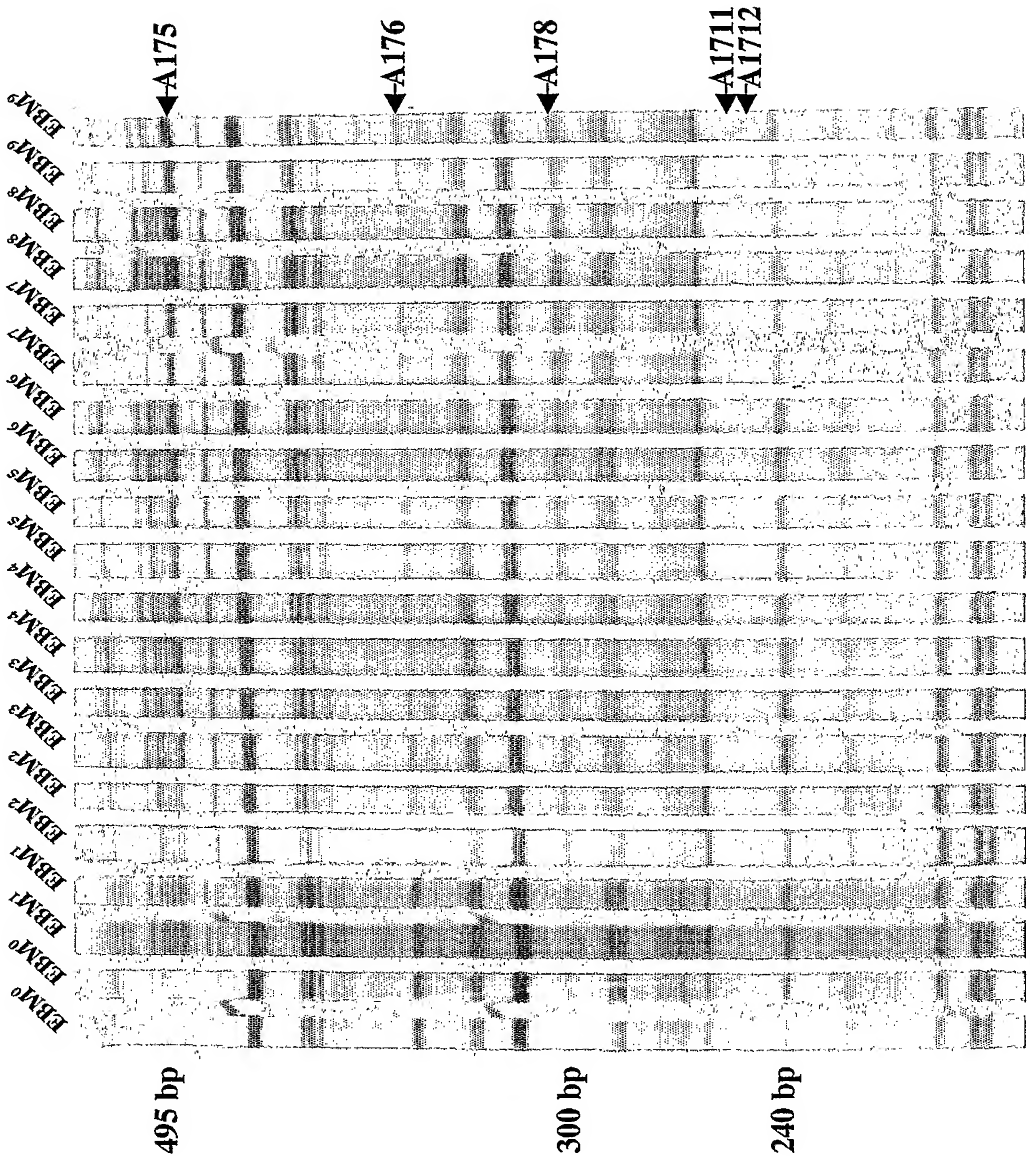
A175
A176
A178
A1711
A1712
EBM9
EBM9
EBM8
EBM8
EBM7
EBM7
EBM6
EBM6
EBM5
EBM5
EBM4
EBM4
EBM3
EBM3
EBM2
EBM2
EBM1
EBM1
EBM0
EBM0
495 bp
300 bp
240 bp

FIGURE 6

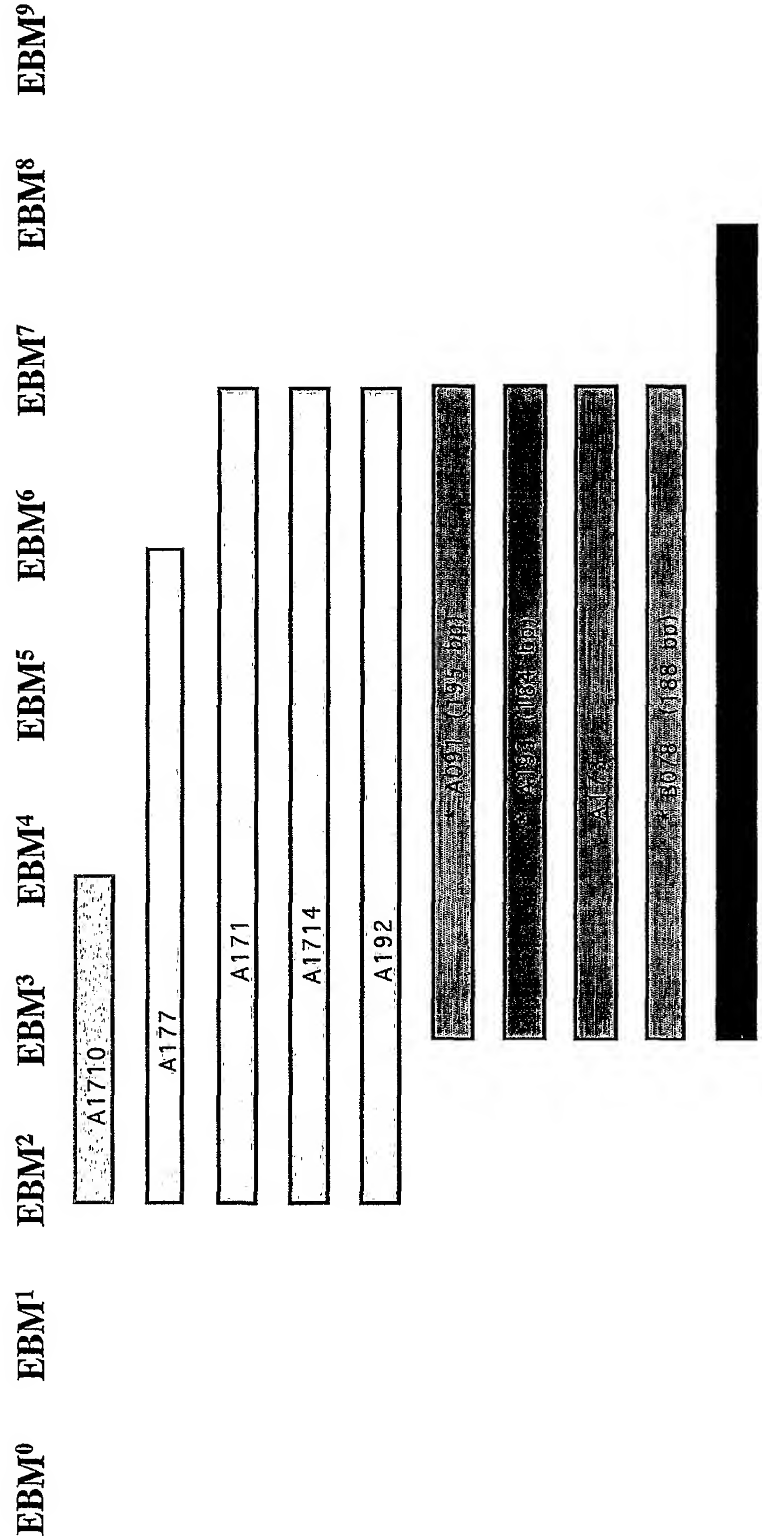
EBM0
EBM1
EBM2
EBM3
EBM4
EBM5
EBM6
EBM7
EBM8
EBM9
A1710
A177
A171
A1714
A192
A091 (195 bp)
A193 (184 bp)
B078 (188 bp)

FIGURE 7

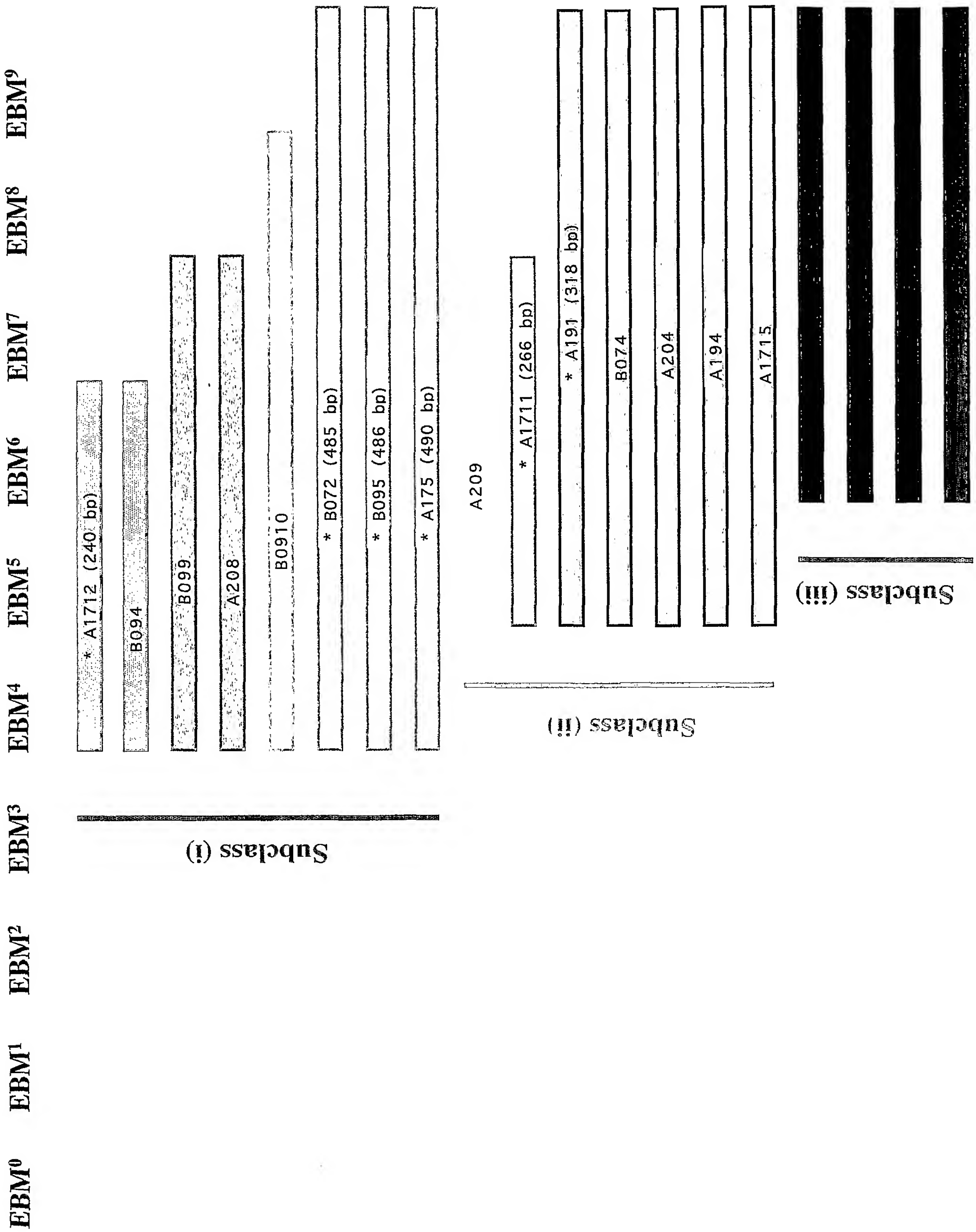
EBM9
EBM8
EBM7
EBM6
EBM5
EBM4
EBM3
EBM2
EBM1
EBM0
* A1712 (240 bp)
B094
B099
A208
B0910
* B072 (485 bp)
* B095 (486 bp)
* A175 (490 bp)
Subclass (i)
A209
* A1711 (266 bp)
* A191 (318 bp)
B074
A204
A194
A1715
Subclass (ii)
Subclass (iii)

FIGURE 8

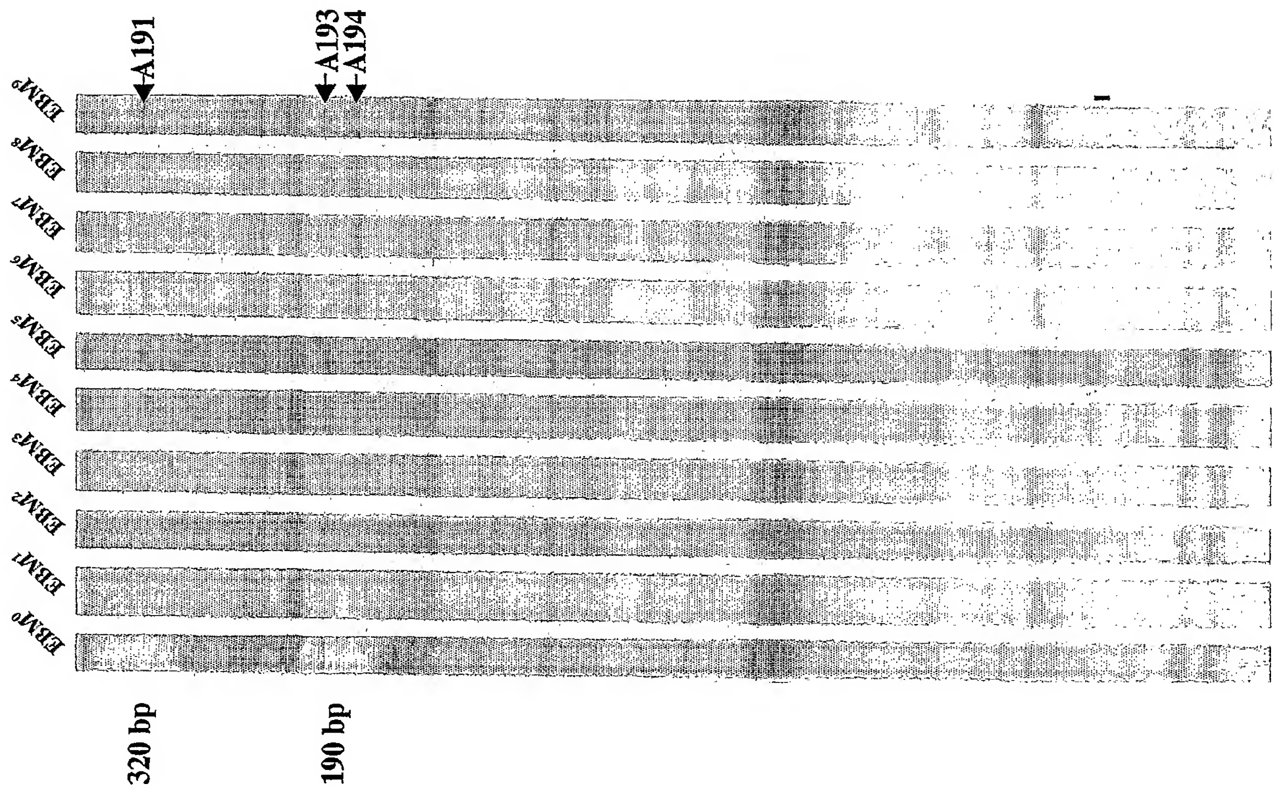
A191
A193
A194
EBM9
EBM8
EBM7
EBM6
EBM5
EBM4
EBM3
EBM2
EBM1
EBM0
320 bp
190 bp

FIGURE 9

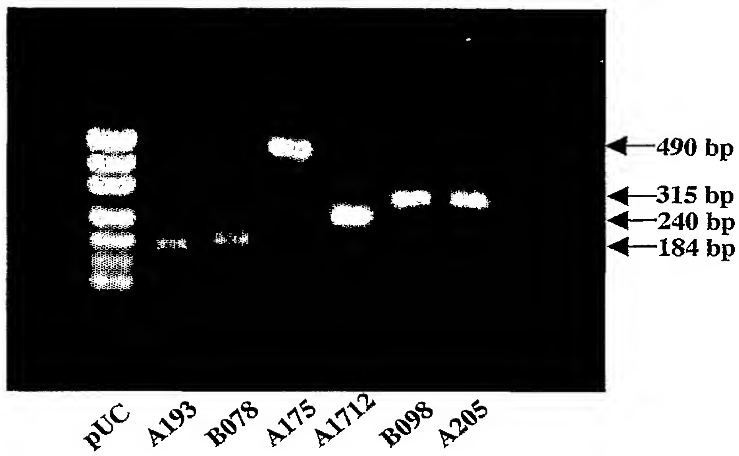

FIGURE 10

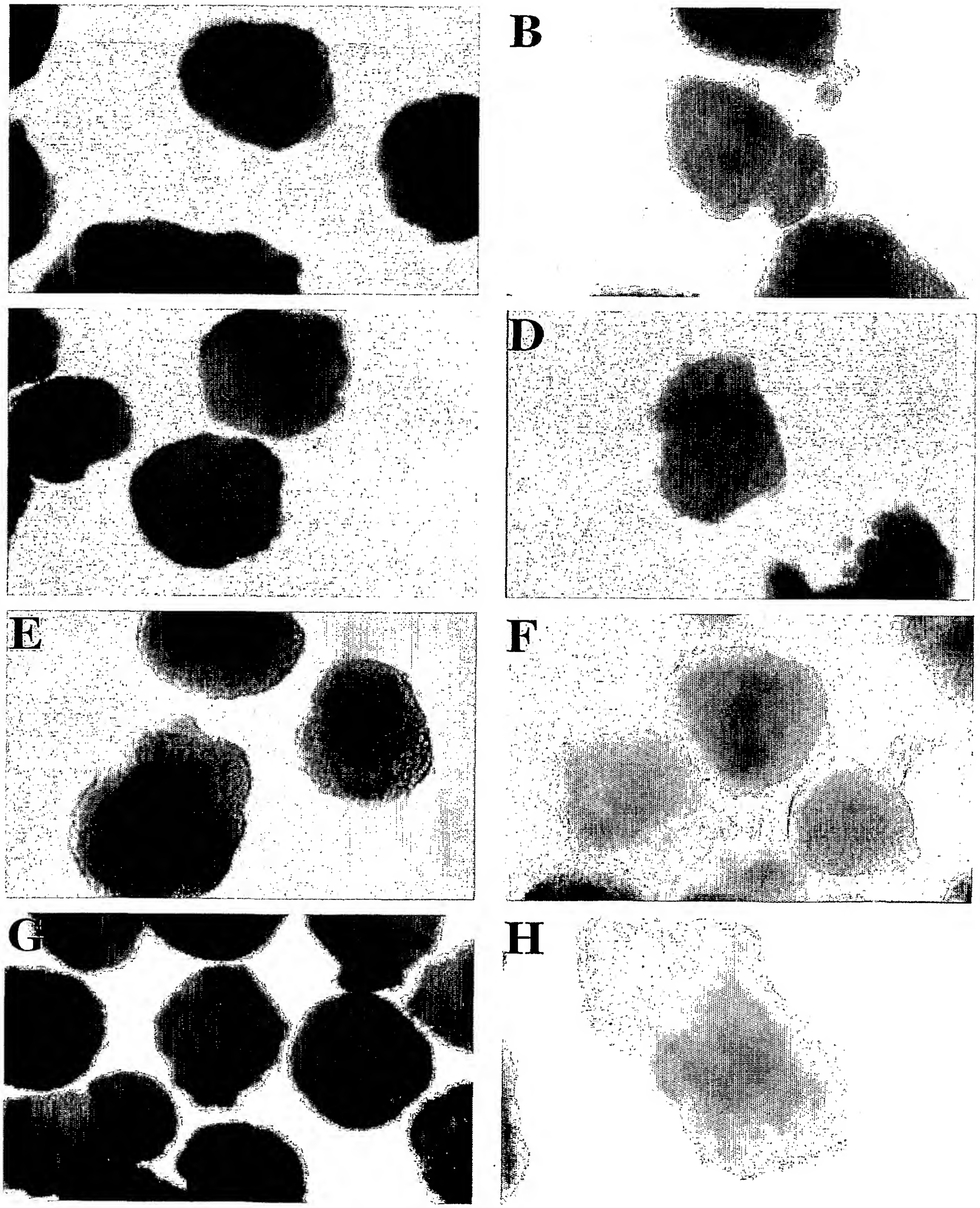

FIGURE 11

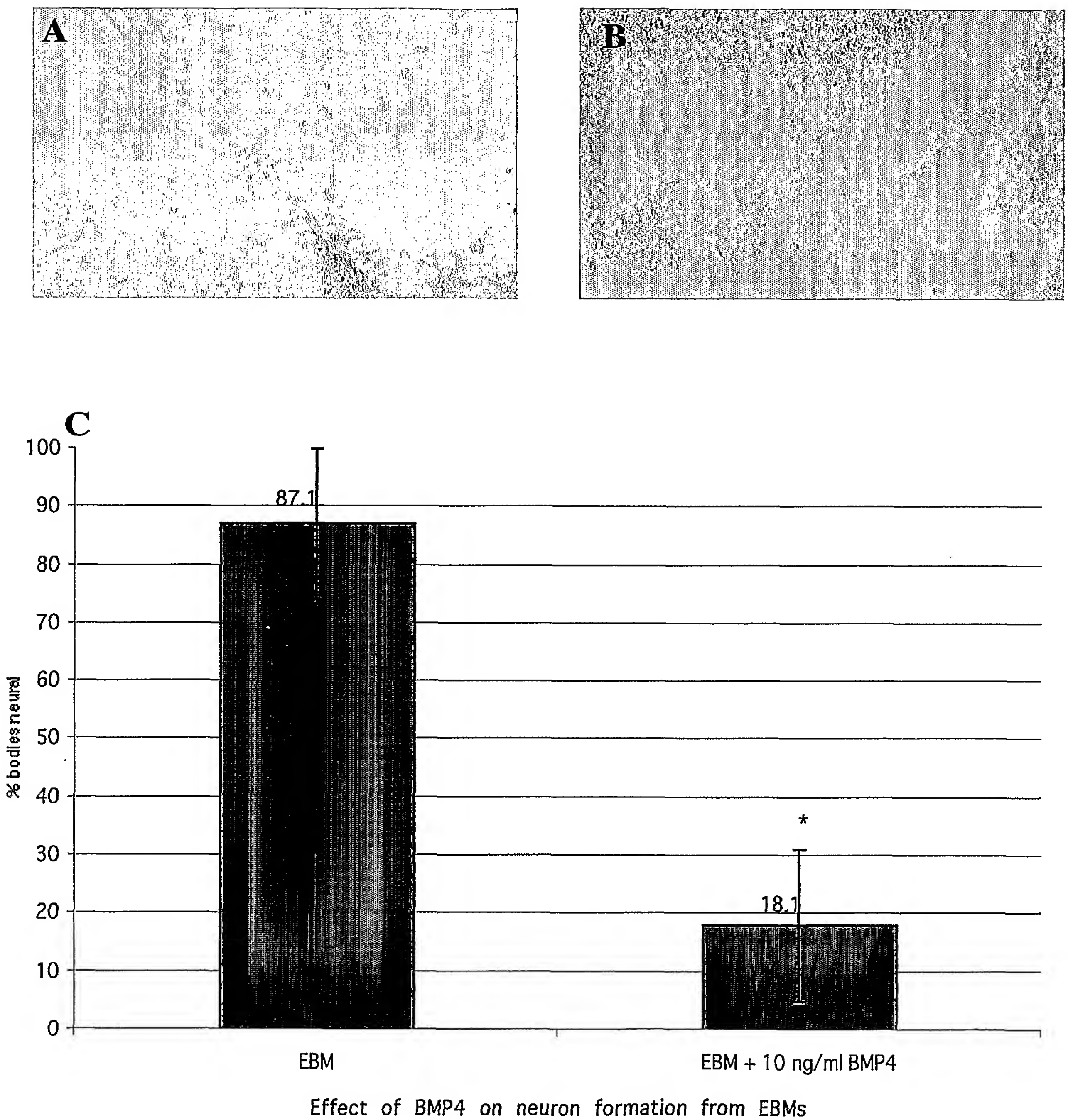
A
B
C
100
90
80
70
60
50
40
30
20
10
0
% bodies neural
87.1
18.1
*
EBM
EBM + 10 ng/ml BMP4
Effect of BMP4 on neuron formation from EBMs

FIGURE 12

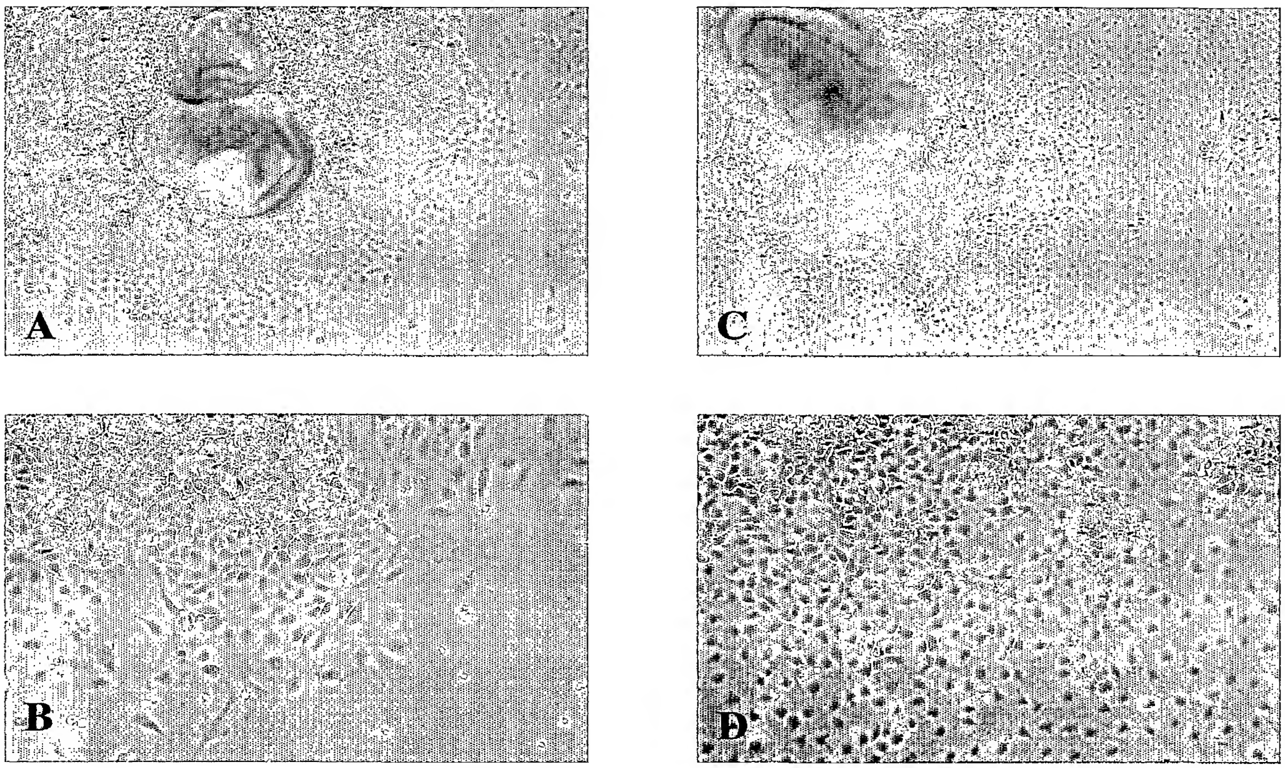

FIGURE 13

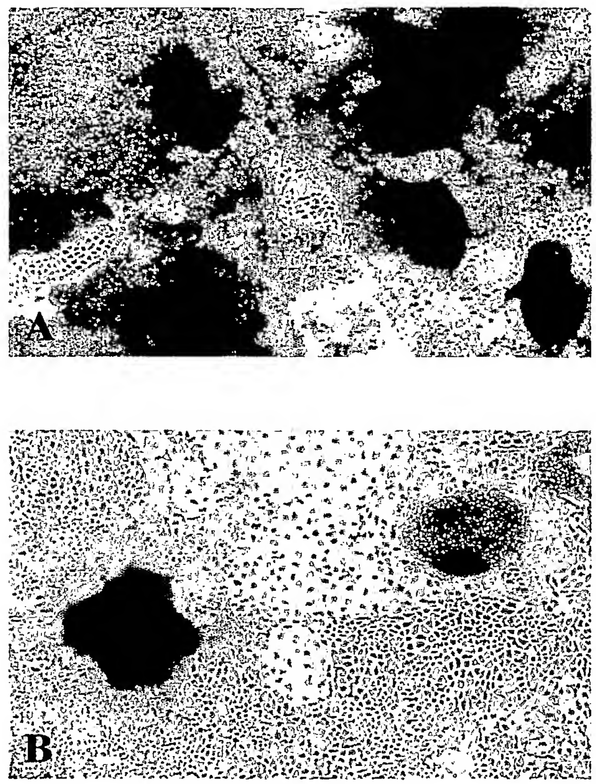

FIGURE 14

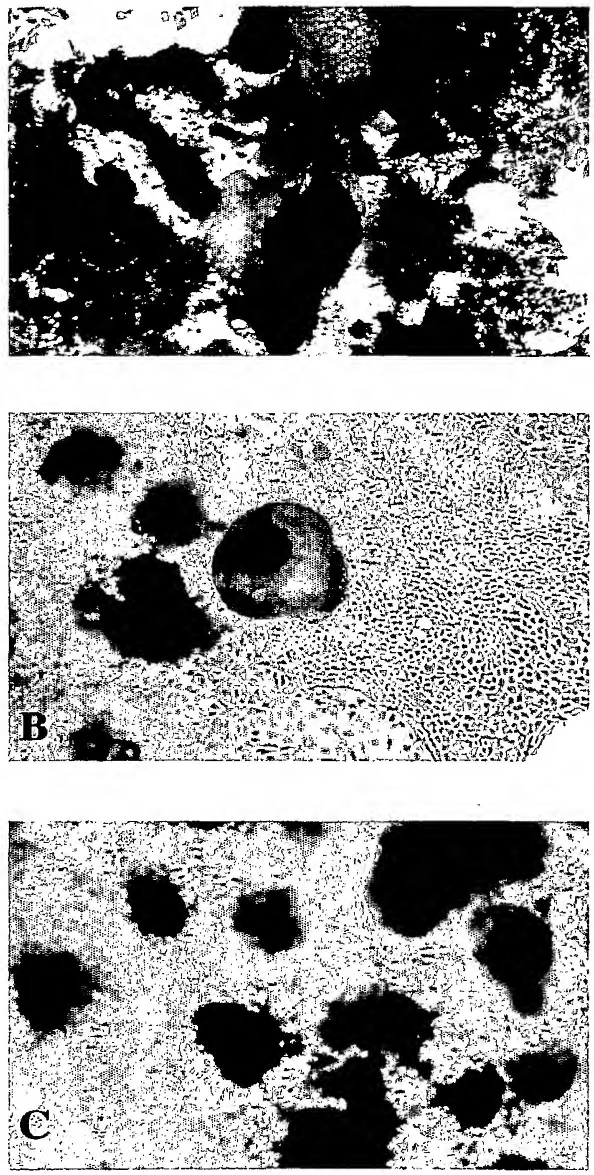

FIGURE 15

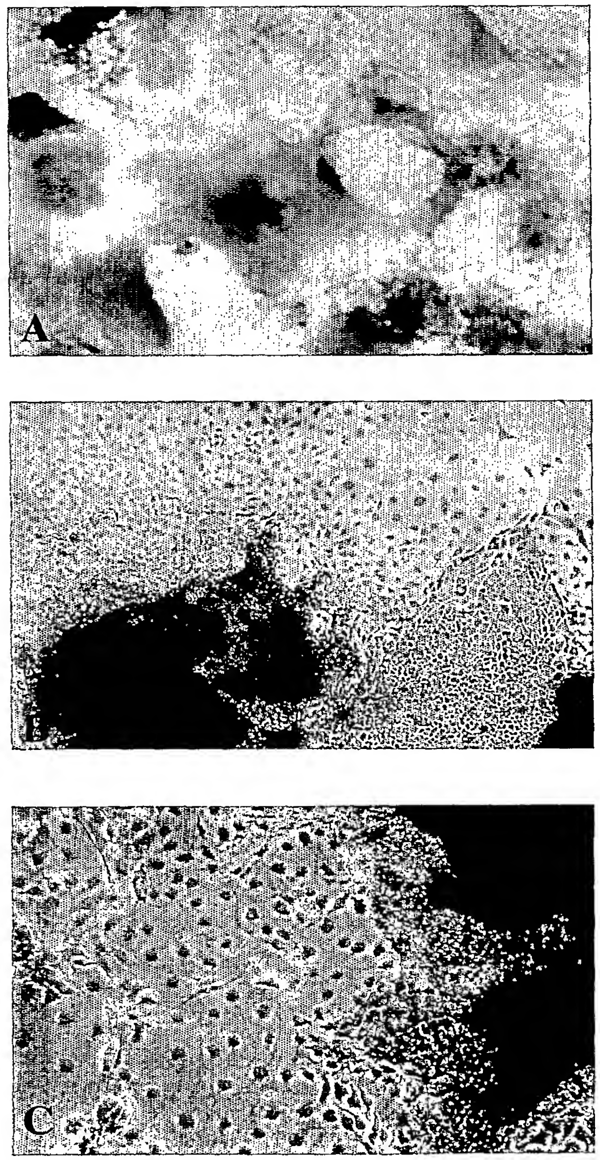

FIGURE 16

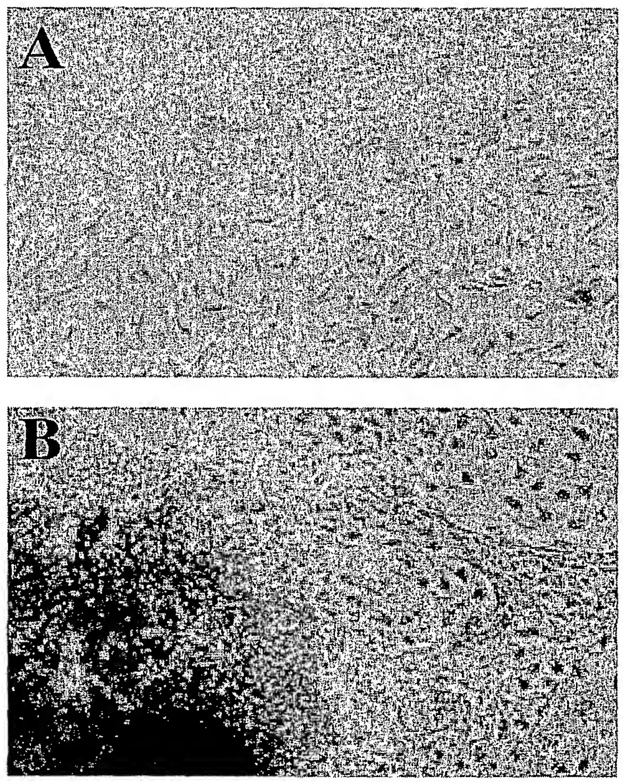

FIGURE 17

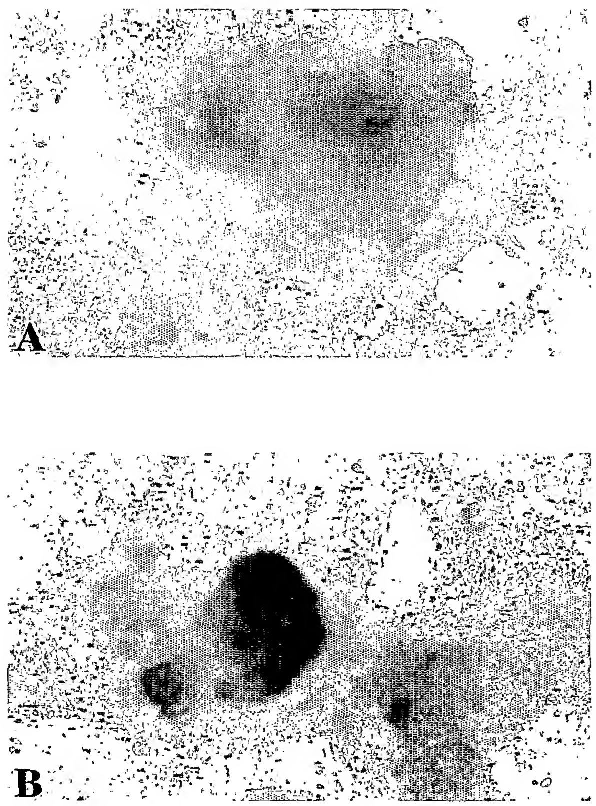

FIGURE 18

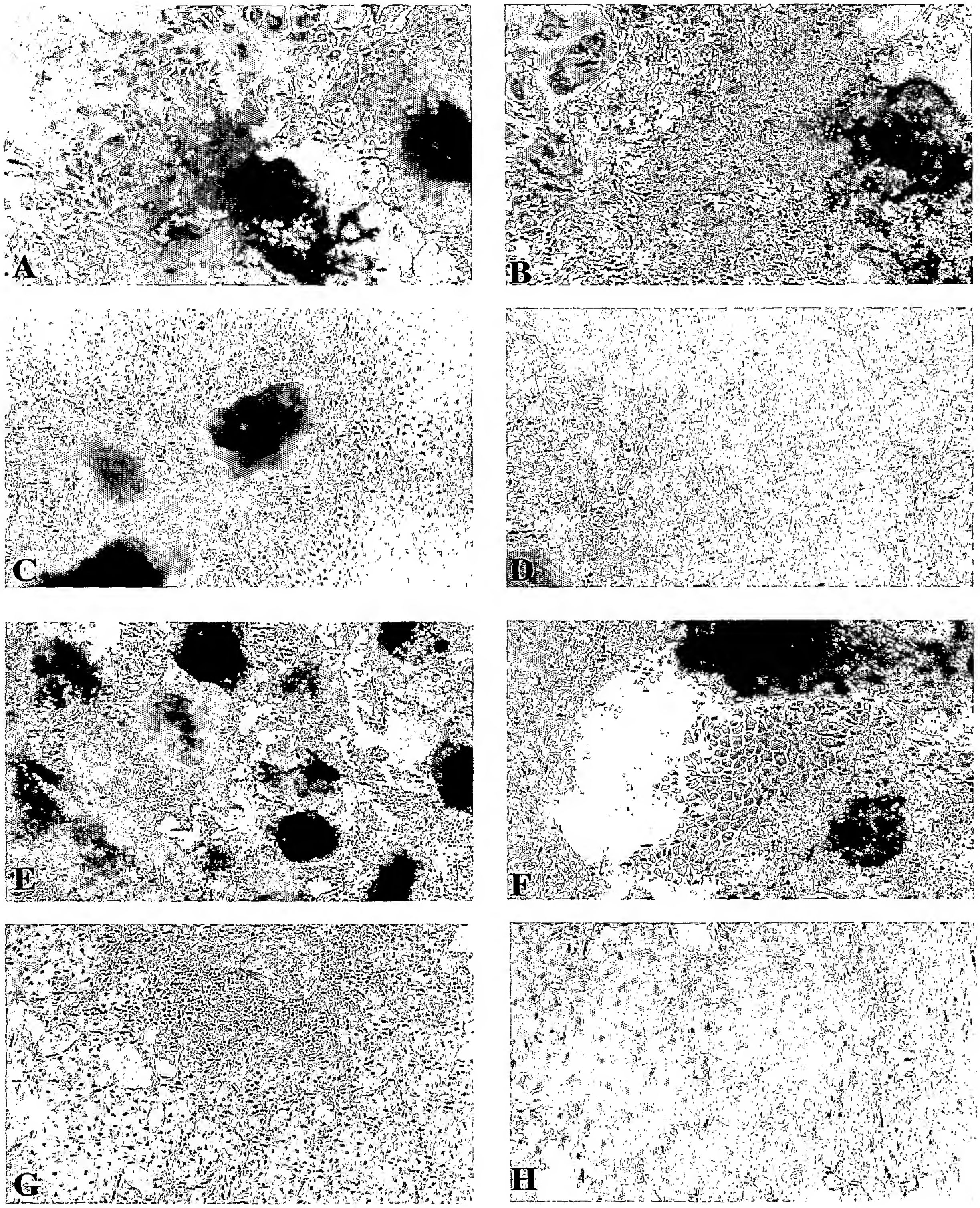

FIGURE 19

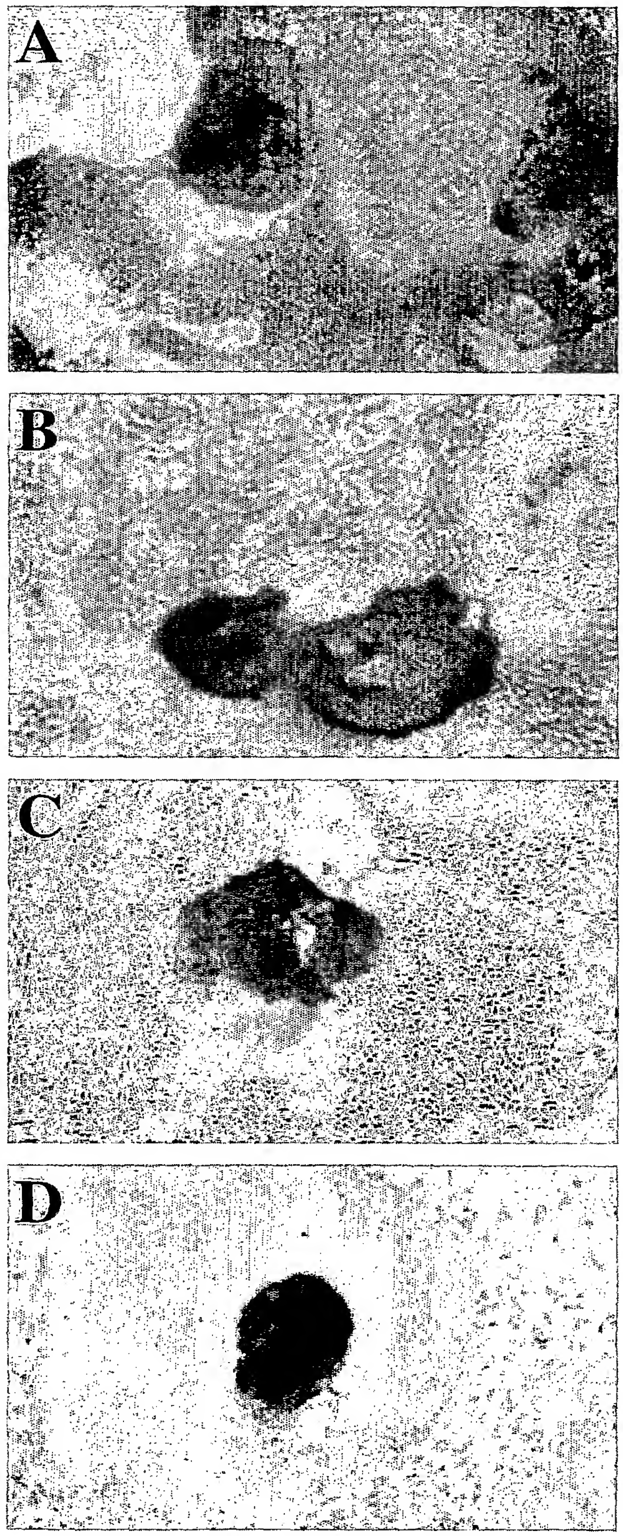

FIGURE 20

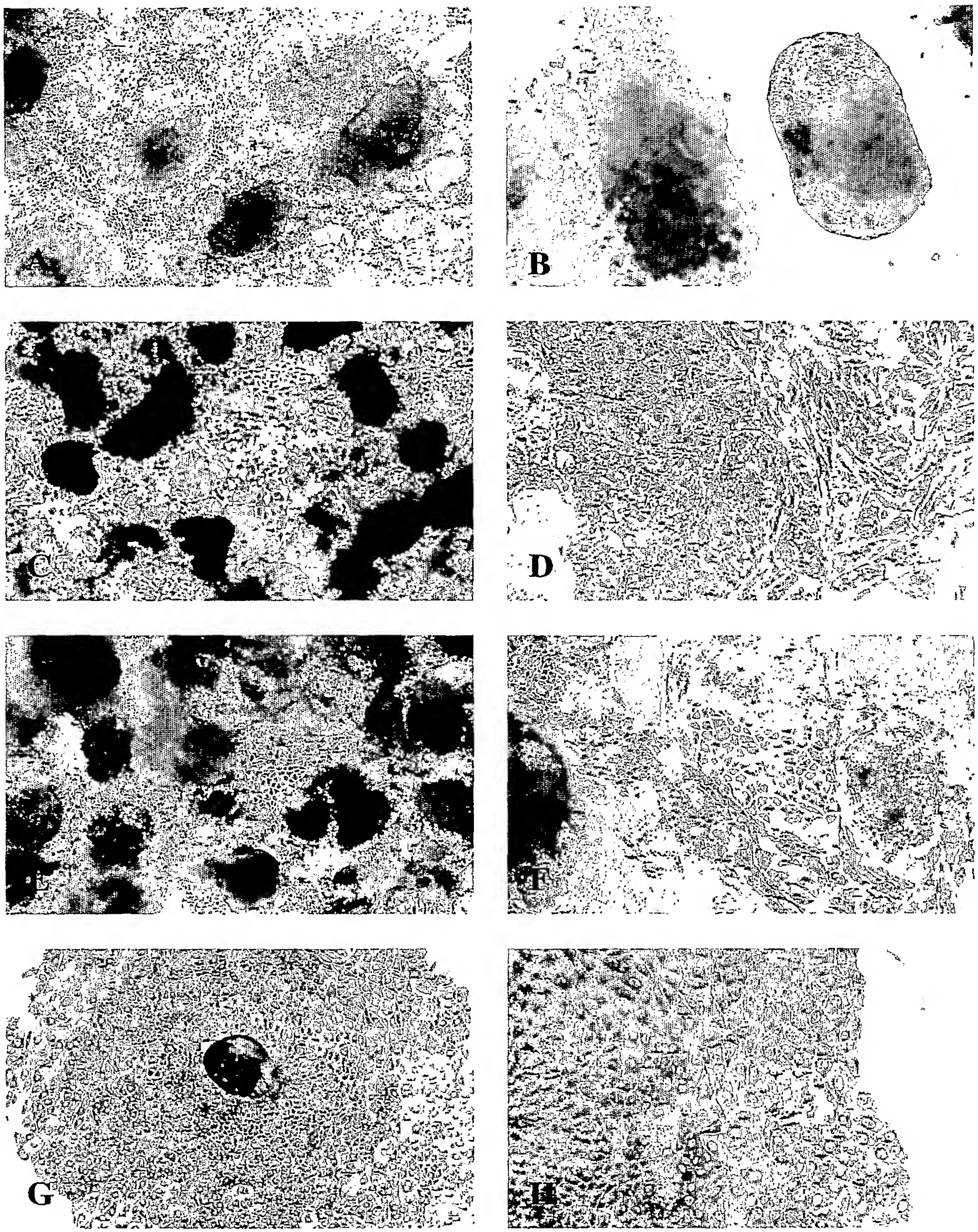

FIGURE 21

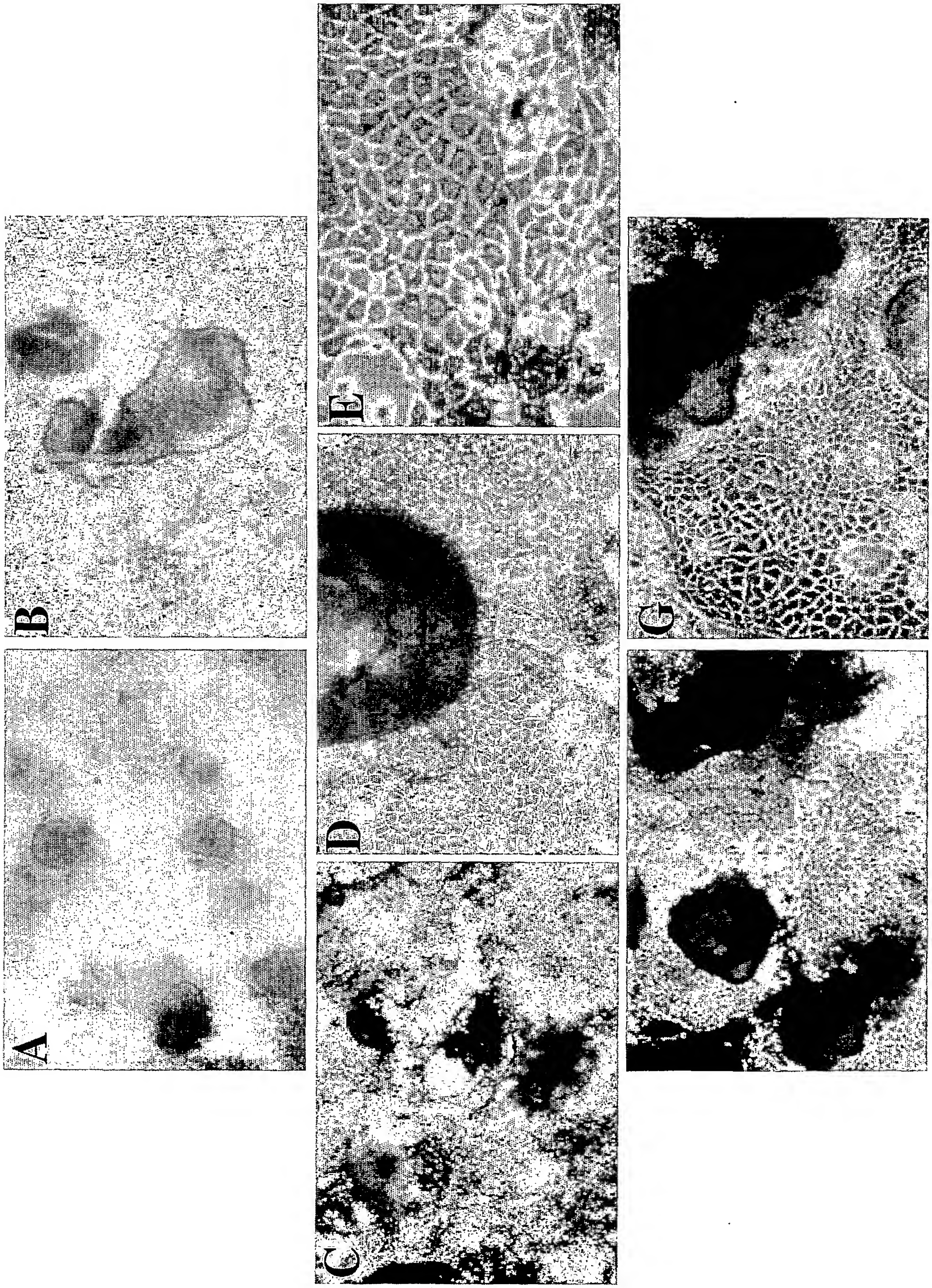

FIGURE 22

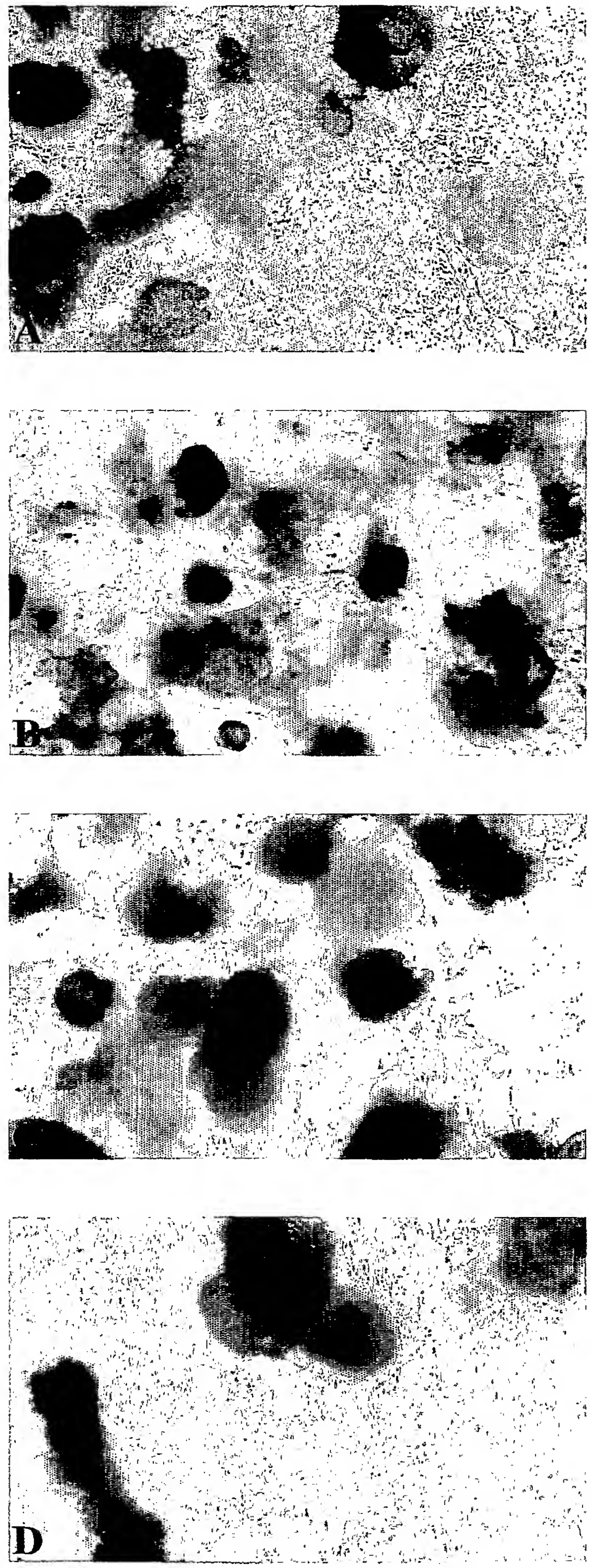

FIGURE 23

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU01/00029

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ^{7}: C12N 5/06, 5/08 C12N 15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

WPIDS, CA: SEE ELECTRONIC DATA BASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

MEDLINE, DNA DATABASES: SEE ELECTRONIC DATA BASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS, CA, MEDLINE, EMBL, GENBANK: SEE SUPPLEMENTARY SHEET

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	**Genome Res 10(10)**, pages 1617-30 (2000) Carninci, P. et al "Normalization and subtraction of cap-trapper-selected cDNAs to prepare full-length cDNA libraries for rapid discovery of new genes." See table 1, relevant to sequences A17113A, A091, A191, A193	11-13
PX	**Cytogenet Cell Genet 90(3-4)**, pages 227-30 (2000) Kleiter, N. et al "Genomic organization and chromosome location of the murine Rpl23 gene." See page 227, column 2, paragraph 2, relevant to Rpl23 sequence	11-13

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2001	20 March 2001

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**TERRY MOORE** Telephone No : (02) 6283 2632

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	**J Cell Science 113**, pages 555-566 (2000) Lake, J-A. et al "Reversible programming of pluripotent cell differentiation." See the entire document.	21
X	**Genbank Accession No X72910** 29 October 1999 *M. musculus* Cd24a gene. See the entire sequence, relevant to CD24a	11-13
X	**WO 9953021 A** (BRESAGEN LIMITED) 21 October 1999 See pages 92, 93, 97-102, figure 34B	21
X	**EMBL Accession No. AF073797** 5 August 1999 *Mus musculus* cosmid MPMGc121L12287 containing the syntenic region of the human AIRE gene, complete sequence. See the sequence, relevant to A1712	11-14
X	**WO 9932606 A** (BRUSTLE, O.) 1 July 1999 See the entire document	21
X	**EMBL Accession No AC004771** 29 September 1998 See the sequence, relevant to A205	11-14
X	**J Biol Chem 273(12)**, pages 6704-9 (1998) Tsuji, T. et al "Cloning, mapping, expression, function, and mutation analyses of the human ortholog of the hamster putative tumor suppressor gene *doc-1*." See figure 2	11-13
X	**Genbank Accession No M14200** 2 November 1994 Human diazepam binding inhibitor (DBI) mRNA, complete cds. See the sequence, relevant to DBI	11-13
A	**J Cell Science 112**, pages 601-12 (1999) Rathjen, J. et al "Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors." See the entire document	1-70

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: B

ELECTRONIC DATA BASES CONSULTED FOR SEARCH AND TERMS USED:

CA, WPIDS, MEDLINE:	cell differentiation, neural crest, glial, ectoderm, neurectoderm, epl, primitive ectoderm, culture media, cell culture
GENBANK, EMBL:	DNA sequences A1712, A17113A, A091, A191, A193, A205

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU01/00029**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	99 53021	AU	33219/99	EP	1068295
WO	99 32606	AU	25106/99	EP	1040185

END OF ANNEX